PHILIPP AGRIC SCIENTIST
Vol. 100 No. 1, 24–36
March 2017

ISSN 0031-7454

Phylogeny and Evolutionary History of *Brassica* Species in China Based on Chalcone Synthase Gene (*Chs*) Sequence

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For many years, relationships within Chinese *Brassica* species and subspecies were the subject of much controversy. Sequences of the chalcone synthase gene (*Chs*) were used to analyze the evolutionary history of *Brassica* plants from China. Sequences from *Brassica* were separated into three well-supported groups in accordance with the A, B, and C genomes. SplitsTree analysis recognized three distinct *Brassica* groups, and median-joining network analysis recognized three distinct haplotypes of *Chs*. The estimates of Tajima's *D*, Fu and Li's *D*, and Fu and Li's *F* statistics for the *Chs* gene between the A-diploid and C-diploid were not significant, while those between the A-polyploid and B-polyploid were significant. The results indicated that (1) Chinese *Brassica* could be divided into three sections – *Pekinensis*, *Juncea*, and *Oleracea*; (2) both tree and reticulate evolution existed in the evolution of Chinese *Brassica*; (3) *B. rapa* var. *oleifera*, *B. nigra*, and *B. oleracea* were the parental donors of the A genome, B genome, and C genome in the allotetraploid, respectively; and (4) the relationship between the A and B genomes was closer than that between the A and C, and B and C genomes in Chinese *Brassica*. These results shed new light on the knowledge about the phylogeny and evolution of *Brassica* in China that could account for rich resources of *Brassica* species.

Key Words: *Brassica*, *Chs* gene, phylogenetic relationship, tetraploid

Abbreviations: BI – Bayesian inference, BS – bootstrap support, *Chs* – chalcone synthase gene, HKA test – Hudson, Kreitman and Aguadé's test, MJ – median-joining, ML – maximum likelihood, PP – posterior probabilities

INTRODUCTION

The *Brassica* genus belongs to the *Brassicaceae* family and includes agriculturally and economically important crops such as cabbage, cauliflower, broccoli, Brussels sprouts, mustard, and rape (Gautam et al. 2014). According to the Triangle of U theory (Nagaharu 1935), there are three basic species: *B. rapa* (2n=AA=20), *B. nigra* (2n=BB=16), and *B. oleracea* (2n=CC=18), while *B. napus* (2n=AACC=38), *B. juncea* (2n=AABB=36), and *B. carinata* (2n=BBCC=34) are allotetraploids evolved from the combination of chromosomes in the three basic species. There are approximately 40 *Brassica* species, of which five, *B. rapa*, *B. nigra*, *B. oleracea*, *B. napus*, and *B. juncea*, are found in China (Wang et al. 2006).

One of the prime centers of origin of *Brassica* is China where rich *Brassica* resources are found (Wang et al. 2006). Because of its importance in agriculture, *Brassica* has been the subject of much scientific interest in China. Data on the origin, evolution, and the genetic diversity of *Brassica* plants derived from molecular markers enable better protection and use of resources, facilitates interspecific hybridization, and allow creation of new *Brassica* crop materials (Chen et al. 2013; Sui et al. 2014).

Under the long-time selections imposed by nature and humans, Chinese *Brassica* has evolved from its original dwarf shape to now include great variations in root, leaf, stem, and seed stalk form. Wang et al. (2006) classified Chinese *Brassica* plants into three groups, *Pekinensis*, *Juncea*, and *Oleracea*, in which *Pekinensis* members are the original species, *Juncea* are more evolved, and *Oleracea* have evolved the most. Meng et al. (2006) divided Chinese *B. juncea* into five different types, consisting of stem

mustard, leaf mustard, root mustard, seed stalk mustard, and seed mustard, based on the characteristics of leaf, root, stem, flower, and seed. Yao et al. (2012), Fang et al. (2013), Liu et al. (2014a), and Xu et al. (2014) have reported on the genetic diversity of Chinese *Brassica*, detected high levels of molecular variation in Chinese *Brassica*, and developed a better understanding of breeding potential.

Results revealed by molecular markers were partially in accordance with the traditional classification based on edible organs. Qi et al. (2007) investigated the molecular phylogeny and probable evolutionary patterns of amphidiploid Chinese vegetable mustards using nuclear internal transcribed spacer regions of ribosomal DNA (ITS1) sequences. They suggested that *B. juncea* is closely related to the A-genome type, and speculated that *B. juncea* crops evolved through different recombination events of diploid morphotypes and evolved unidirectional concerted evolution. Despite these studies, little is known about the evolutionary history of Chinese *Brassica* species, especially the polyploid molecular evolution of the allotetraploids in *Brassica*. Therefore the main purpose of this study is to reconstruct phylogenetic relationships among *Brassica* species at the molecular level.

Recently single- and low-copy genes have received increasing attention in plant evolution studies, and have become ideal tools for studying the origin and evolution of polyploid taxa (Hochbach et al. 2015). The chalcone synthase gene (*Chs*) is widespread in plants as a single low-copy gene that encodes the first enzyme in the plant flavonoid biosynthesis pathway (Bao et al. 2015). *Chs* is an excellent marker for analysis of the origin of polyploids as: (1) *Chs* is well conserved in different plant species (Abe and Morita 2010); (2) it has provided high confidence in reconstructions, particularly at deeper nodes (Bao et al. 2015); and (3) it has biparental inheritance and is highly variable (Zhao et al. 2010). In this study, we analyzed the molecular phylogenetic relationships of Chinese *Brassica* species and subspecies using data from single-copy nuclear gene *Chs* sequences, in order to (i) estimate the *Chs* nucleotide polymorphism of Chinese *Brassica*; (ii) elucidate phylogenetic relationships among Chinese *Brassica* species and subspecies; and (iii) infer maternal donors and relationships among the A-genome, B-genome, and C-genome in *Brassica*.

MATERIALS AND METHODS

Plant Materials

We sampled 72 individuals (11 *B. rapa*, 1 *B. nigra*, 13 *B. oleracea*, 2 *B. napus*, 38 *B. juncea*, 1 *B. carinata*, and 6 *Raphanus sativus*) as out-group (Table 1). The species were chosen on the basis of genetic relationships between Chinese *Brassica* according to the classifications made by Wang et al. (2006). The seeds and voucher specimens were obtained from those that are deposited at the herbarium of the Crop Genetics and Breeding Research Centre, Yangtze Normal University, China.

DNA Amplification and Sequencing

Total genomic DNA was extracted from the fresh young leaf (Doyle and Doyle 1987). The first and second exons (about 1200 bp) were amplified with the *Chs*-specific primers. The homologous sequences EF408922 and GQ983033 representing *B. rapa* and *B. nigra*, respectively, were loaded from GenBank (http://www.ncbi.nlm.nih.gov/) for primer design, and the primers were designed by the software Primer 5 (Lalitha 2004).

The DNA sequence for the common forward primer was 5'- CTT CAT CTG CCC GTC CAT CAT ACC - 3', and the sequence for the common reverse primer was 5'-GGAACGCTGTGCAAGAC-3'. The primers were synthesized by Yinggen Bio-Tech in Shanghai City, China. Polymerase chain reaction (PCR) was performed in a reaction mixture (25 µL) containing 12.5 µL 2×Taq Master Mix (Kuangweishiji Biotechnology, Beijing, China), 2 µL of each primer (10 nmol/mL), 1 µL (50 ng) DNA, and 7.5 µL RNase Free Water. The Mastercycle Personal PCR System (Eppendorf, German) was programmed to run for 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final extension phase of 10 min at 72 °C.

After electrophoresis of the PCR products in a 1.0% agarose gel, a single band of amplified products was cut out and purified with a gel extraction kit (AxyPrep Bio-Tech, Huangzhou, China). The purified DNA fragments were cloned into pMD18-T vector (TaKaRa, Huangzhou, China). At least five positive clones for the species were randomly selected for sequencing. The positive clones were sequenced by Yinggen Bio-Tech Company (Shanghai, China). In the case of multiple identical sequences in an individual by

Table 1. Materials used in the study and their source.

No.	Name	Type	Chromo-somes	Latin Name	Source	Accession No.
1	Yaan Cabbage	Chinese Cabbage	AA	*B. rapa* var. *pekinensis*	Yaan, Sichuan, China	KP301145
2	Shandong Cabbage	Chinese Cabbage	AA	*B. rapa* var. *pekinensis*	Shandong, Jinan, China	KP301146
3	Chongqing Cabbage	Chinese Cabbage	AA	*B. rapa* var. *pekinensis*	Yubei, Chongqing, China	KP301147
4	Purple Caitai	flowering cabbage	AA	*B. rapa* var. *purpuraria*	Yaan, Sichuan, China	KP301148
5	Takecai	pakchoi	AA	*B. rapa* var. *narinosa*	Yaan, Sichuan, China	KP301149
6	Qingcai	Chinese Cabbage	AA	*B. rapa* var. *Chinensis* L.	Xichang, Sichuan, China	KP301150
7	Guangxicaixin	flowering cabbage	AA	*B. rapa* var. *parachinensis*	Guilin, Guangxi, China	KP301151
8	Wuqing	turnip	AA	*B. rapa* var. *rapa* L.	Yaan, Sichuan, China	KP301152
9	Yaanhuangyoucai	turnip type rape	AA	*B. rapa* var. *oleifera*	Yaan, Sichuan, China	KP301153
10	Yiliyueyoucai	wild rape	AA	*B. rapa* var. *sinapis arvensis* Tsen et Lee	Yili, Xinjiang, China	KP301155
11	Fulingyueyoucai	wild rape	AA	*B. rapa* var. *oleifera*	Fuling, Chongqing, China	KP301156
12	Heijie	black mustard	BB	*B. nigra* L.	Yili, Xinjiang, China	KP301157
13	Shandongganlan	head cabbage	CC	*B. oleracea* var. *capitata* Léveillé	Shandong, Jinan, China	KP301158
14	Xiyuan4	head cabbage	CC	*B. oleracea* var. *capitata* Léveillé	Beibei, Chongqing, China	KP301159
15	Yuyiganlan	white kale	CC	*B. oleracea* var. *acephala* Hort	Beijing, China	KP301160
16	Ziganlan	purple kale	CC	*B. oleracea* var. *acephala* Hort	Yaan, Sichuan, China	KP301161
17	Yaan Cauliflower	cauliflower	CC	*B. oleracea* var. *botrytis* Linnaeus	Yaan, Sichuan, China	KP301162
18	Zhongqing8	cauliflower	CC	*B. oleracea* var. *botrytis* Linnaeus	Beijing, China	KP301163
19	Yidaitaimei	cauliflower	CC	*B. oleracea* var. *botrytis* Linnaeus	Chongqing, China	KP301164
20	Baoziganlan	Brussels sprout	CC	*B. oleracea* var. *gemmifera*	Holland	KP301165
21	Wuqingganlan	winter rape	CC	*B. oleracea* var. *napobrassica* L. H. Bailey	Xichang, Sichuan, China	KP301166
22	Panzhihuajielan	cabbage mustard	CC	*B. oleracea* var. *alboglabra* L. H. Bailey	Panzhihua, Sichuan, China	KP301167
23	Baihua jielan	cabbage mustard	CC	*B. oleracea* var. *alboglabra* L. H. Bailey	Guangzhou, Guangdong, China	KP301168
24	Peilan	kohlrabi	CC	*B. oleracea* var. *gongylodes* L. H. Bailey	Xingtai, Hebei, China	KP301169
25	Purple Peilan	kohlrabi	CC	*B. oleracea* var. *gongylodes* L. H. Bailey	Xingtai, Hebei, China	KP301170
26	Zhongyou1 rape	rapeseed	AACC	*B. napus* L.	Beijing, China	KP301171, KP301172
27	Du06rape	rapeseed	AACC	*B. napus* L.	Germany	KP301173, KP301174
28	Y8	wild rape	AABB	*B. juncea* var. *juncea* Tsen et Lee	Jiuquan, Ganshu, China	KP301175, KP301242
29	Zigongdatoucai	root mustard	AABB	*B. juncea* var. *megarrhiza* Tsen et Lee	Zigong, Sichuan, China	KP301176, KP301243
30	Wanyuandatoucai	root mustard	AABB	*B. juncea* var. *megarrhiza* Tsen et Lee	Wanyuan, Sichuan, China	KP301177, KP301244
31	Neijiangbangcai	stem mustard	AABB	*B. juncea* var. *carassicaulis* Chen et Yang	Niejiang, Sichuan, China	KP301178, KP301179
32	Baijiacaitai	stem mustard	AABB	*B. juncea* var. *carassicaulis* Chen et Yang	Zigong, Sichuan, China	KP301180, KP301245
33	Chuannong1	stem mustard	AABB	*B. juncea* var. *gemmifera* Lee et Li	Yaan, Sichuan, China	KP301181, KP301247
34	Dianjiangbaoercai	stem mustard	AABB	*B. juncea* var. *gemmifera* Lee et Li	Dianjiang, Chongqing, China	KP301182, KP301246
35	Pixian zhacai	stem mustard	AABB	*B. juncea* var. *tumida* Tsen et Lee	Pixian, Sichuan, China	KP301183, KP301184
36	Linshichaoyaozi	stem mustard	AABB	*B. juncea* var. *tumida* Tsen et Lee	Fuling, Chongqing, China	KP301185, KP301186
37	Aihelingqingcai	stem mustard	AABB	*B. juncea* var. *tumida* Tsen et Lee	Bazhong, Sichuan, China	KP301187, KP301188
38	Yonganxiaoye	stem mustard	AABB	*B. juncea* var. *tumida* Tsen et Lee	Fuling, Chongqing, China	KP301189. KP301248

Table 1. Continued. . .

No.	Name	Type	Chromo-somes	Latin Name	Source	Accession No.
39	Huanghacai	stem mustard	AABB	*B. juncea* var. *tumida* Tsen et Lee	Fuling, Chongqing, China	KP301190, KP301191
40	Zhetongyihao	stem mustard	AABB	*B. juncea* var. *tumida* Tsen et Lee	Yuyao, Zhejiang, China	KP301192, KP301249
41	Dongcai	leaf mustard	AABB	*B. juncea* var. *rugosa* Bailey	Dazhu, Chongqing, China	KP301193, KP301194
42	Midulvgan	leaf mustard	AABB	*B. juncea* var. *rugosa* Bailey	Midu, Yunnan, China	KP301195, KP301196
43	Baiganqingcai	leaf mustard	AABB	*B. juncea* var. *foliosa* Bailey	Luzhou, Sichuan, China	KP301197, KP301198
44	Zhayetianqingcai	leaf mustard	AABB	*B. juncea* var. *foliosa* Bailey	Mabian, Sichuan, China	KP301199, KP301200
45	Baihuacai	leaf mustard	AABB	*B. juncea* var. *leucanthus* Chen et Yang	Luxian, Sichuan, China	KP301201, KP301202
46	Baihuaqingcai	leaf mustard	AABB	*B. juncea* var. *leucanthus* Chen et Yang	Luxian, Sichuan, China	KP301203, KP301250
47	Huayejiecai	leaf mustard	AABB	*B. juncea* var. *multisecta* Bailey	Ezhou, Hubei, China	KP301204, KP301252
48	Huayejiecai	leaf mustard	AABB	*B. juncea* var. *multisecta* Bailey	Nanchuang, Jiangxi, China	KP301205, KP301251
49	Liangpingxiangcai	leaf mustard	AABB	*B. juncea* var. *longepetiolata* Yang et Chen	Liangping, Chongqing, China	KP301206, KP301253
50	Fengduxiangcai	leaf mustard	AABB	*B. juncea* var. *longepetiolata* Yang et Chen	Fengdu, Chongqing, China	KP301207, KP301208
51	Yanjiweilacai	leaf mustard	AABB	*B. juncea* var. *linearifolia* Sun	Xichang, Sichuan, China	KP301209 , KP301210
52	Kuanyefengweicai	leaf mustard	AABB	*B. juncea* var. *linearifolia* Sun	Zigong, Sichuan, China	KP301211, KP301212
53	Nainaicai	leaf mustard	AABB	*B. juncea* var. *strumata* Tsen et Lee	Luxian, Sichuan, China	KP301213, P301214
54	Daerduoqingcai	leaf mustard	AABB	*B. juncea* var. *strumata* Tsen et Lee	Yuanjiang, Hunan, China	KP301215, KP301254
55	Dapianpianqingcai	leaf mustard	AABB	*B. juncea* var. *latipa* Li	Meigu, Sichuan, China	KP301216, KP301217
56	Baiyeqingcai	leaf mustard	AABB	*B. juncea* var. *latipa* Li	Zigong, Sichuan, China	KP301218, KP301255
57	Qingyebaobaocai	leaf mustard	AABB	*B. juncea* var. *involuta* Yang et Chen	Dianjiang, Chongqing, China	KP301219, KP301220
58	Baoxinqingcai	leaf mustard	AABB	*B. juncea* var. *involuta* Yang et Chen	Dazhou, Sichuan, China	KP301221, KP301256
59	Jixinjiecai	leaf mustard	AABB	*B. juncea* var. *capitata* Hort	Chaozhou, Guangdong, China	KP301222, KP301223
60	Duanyejixinjiecai	leaf mustard	AABB	*B. juncea* var. *capitata* Hort	Chenghai, Guangzhou, China	KP301224, KP301225
61	Dukexuelihong	leaf mustard	AABB	*B. juncea* var. *multiceps* Tsen et Lee	Nantong, Jiangsu, China	KP301226, KP301227
62	Heiyexuelihong	leaf mustard	AABB	*B. juncea* var. *multiceps* Tsen et Lee	Shanghai, China	KP301228, KP301257
63	Guizhoulacai	seed stalk mustard	AABB	*B. juncea* var. *utilis* Li	Guiyang, Guizhou, China	KP301229, KP301230
64	Xiaoyechonglacai	seed stalk mustard	AABB	*B. juncea* var. *utilis* Li	Banan, Chongqing, China	KP301231, KP301232
65	Maweisi	seed mustard	AABB	*B. juncea* Czern. et Coss.	Suining, Sichuan, China	KP301233, KP301258
66	Aisaiebiyajie	Ethiopia mustard	BBCC	*B. carinata* Braun	Ethiopia	KP301234, KP301235
67	Yuanmoubailuobo	radish	RR	*R. sativus* Linn.	Yuanmou, Yunnan, China	KP301236
68	Dianjiangbailuobo	radish	RR	*R. sativus* Linn.	Dianjiang, Chongqing, China	KP301237
69	Hongluobo	radish	RR	*R. sativus* Linn.	Mianyang, Sichuan, China	KP301238
70	Hongpiluobo	radish	RR	*R. sativus* Linn.	Liupanshui, Guizhou, China	KP301239
71	Hongxinluobo	radish	RR	*R. sativus* Linn.	Fuling, Chongqing, China	KP301240
72	Yanzhiluobo	radish	RR	*R. sativus* Linn.	Fengdu, Chongqing, China	KP301241

DNAman6. 0 (Lynnon Biosoft, Qc, Canada), only one sequence was used for the data set. After the 113 DNA sequences of *Chs* genes were cloned and sequenced, they were accessed to GenBank.

Cloning of PCR amplicons from single-copy nuclear genes from allopolyploid species will isolate homologous sequences from each nuclear genome. In allopolyploid species, the software DNAman 6. 0 (Lynnon Biosoft, Qc, Canada) was used to align and analyze the type of the sequence from A, B, or C genome. Then the specific primers for A and B genomes were designed by using the software Primer 5 (Lalitha 2004). The specific primers for A and B were PA(R: 5'-GCA TTG ATC AAC CTC TTG TAA CT-3', F: 5'-GGA ACG CTG TGC AAG AC-3') and PB(R: 5'-TTG CAT AAA GTC ACA CAT CC-3', F: 5'-GGA ACG CTG TGC AAG AC-3'), respectively.

Phylogenetic Analysis

With maximum likelihood (ML) and Bayesian inference (BI), phylogenetic analysis was carried out using exon + intron data matrixes. ML analysis of the exon + intron data set was conducted using PAUP*4.0 (Swofford 2002). The out-group was *R. sativus* and the evolutionary model used for the data set was determined by ModelTest v3.0 with Akaike information criterion (AIC) (Darriba et al. 2012). The best-fit models were GTR + G for the data set. ML heuristic searches were performed with tree bisection-reconnection branch swapping and 500 random sequence addition replicates. The bootstrap support (BS) was used to estimate the topological robustness of the ML trees. Bootstrap analysis was carried out with 500 replications using simple taxon addition.

With MrBayes v3.2 (Ronquist et al. 2012), BI (Bayesian inference) analysis of *Chs* was conducted. Using MrBayes default heating values (t = 0.2), sampled every 100 generations for a total of 4,000,000 generations, four chains of the Markov Chain Monte Carlo (MCMC) were simultaneously run. The first 18,700 trees were "burned in" the chains and discarded. To ensure that log likelihoods were in the stationary "fury caterpillar" phase, the program Tracer v1.4 (Rambaut and Drummond 2007) was used to test them. The majority rule consensus trees were established on the basis of the remaining trees. Two independent runs were conducted to examine convergence on the same posterior distribution, and the statistical confidence in nodes was estimated using posterior probabilities (PP).

Splits Tree Analyses

To detect reticulate evolution in *Brassica juncea*, we followed the pattern of inferring phylogenetic trees by SplitsTree 4.13 using the neighbor-net method (Huson and Bryant 2006).

Network Analyses

Relationships between haplotypes of taxa sampled can be analyzed by phylogenetic network reconstruction. The median-joining (MJ) network method was used in this study due to its robustness compared with other network methods with known gene phylogenies in a simulation study (Cassens et al. 2005). MJ network was yield using the Network 4.6.1.3 program (Fluxus Technology Ltd., Clare, Suffolk, UK). The test of recombination was performed in HYPHY, version 0.99 (Pond et al. 2005), since median-joining networks are inferred from non-recombining DNA. Based on non-recombining signal in alignment, the exon data was only used to reconstruct MJ network (−Log Likelihood = 3152.23; AIC = 2531.17).

Nucleotide Diversity Estimate

To evaluate nucleotide diversity of A and B genomes in Chinese *Brassica*, sequence variations in *Chs* were estimated by Tajima's $\hat{\pi}$, Watterson's $\hat{\theta}$, and the number of shared polymorphisms and fixed differences. Tajima's $\hat{\pi}$ quantifies mean pairwise differences between sequences, whereas Watterson's $\hat{\theta}$ refers to an index of the number of polymorphic sites (Librado and Rozas 2009). Both $\hat{\pi}$ and $\hat{\theta}$ have expected values of 4 Nμ, where N is the population size and μ is the mutation per locus per generation.

A fixed difference is a site where all sequences sampled in a taxon have a base while those in another taxon have one other base. Shared polymorphisms refer to those in which two taxa have the same two bases segregating at the same site. A test of the neutral evolution model, including Tajima's and Fu and Li's D statistic, was carried out using the methods of Tajima and Fu and Li (Librado and Rozas 2009). The Hudson, Kreitman and Aguadé's (1987) test (HKA test) helped to detect the inter-group genetic evolution. The parameters earlier mentioned were computed with DnaSP v5 (Librado and Rozas 2009).

RESULTS

Chs Phylogenetic Analyses

Two copies of ancestral allelic *Chs* gene types were successfully cloned in each tetraploid species, while three copies of ancestral allelic *Chs* gene types were obtained in all *Brassica*.

ML analysis based on the exon and intron data set resulted in a single phylogenetic tree (−Log likelihood = 2840.26) when using the following ML parameters: inferred nucleotide frequencies A: 0.2434, G: 0.2639, T: 0.2273, C: 0.2654; gamma distribution with shape parameter k = 0.3041; and ratio of invariable sites = 0.1423. A similar topology was revealed in ML and BI analyses. The ML tree with posterior probabilities (PP) above, and bootstrap support (BS) below a branch is displayed in Fig. 1. Sequences from *Brassicaceae* were separated into two well-supported groups (group I and group II); the sequences from *R. sativus* yielded a distinct group (group I). Group II comprised sequences from *B. rapa*, *B. nigra*, *B. oleracea*, *B. napus*, *B. juncea*, and *B. carinata* and included three clades (clade A, clade B, and clade C) with well-defined statistical support; this was in accordance with the A, B, and C genome revealed by *Chs*. Clade C contained sequences from the C-genome that included C1 and C2 subclades (99% PP, 85% BS). Subclade C1 consisted of eight *B. oleracea* and one *B. carinata* (83% PP, 67% BS). Subclade C2 consisted of six *B. oleracea* and two *B. napus* (99% PP, 95% BS). Clade A contained sequences from the A-genome that included five subclades: A1, A2, A3, A4 and A5 (83% PP, 79% BS). Subclade A1 consisted of five *B. rapa* (94% PP, 85% BS). Subclade A2 consisted of four *B. rapa* and 12 *B. juncea* (97% PP, 88% BS). Subclade A3 consisted of one *B rapa*, two *B. napus*, and four *B. juncea* (99% PP, 85% BS). Subclade A4 contained two *B. rapa* and 22 *B. juncea* (92% PP, 89% BS). Subclade A5 consisted of one *B. juncea*. Clade B contained sequences from the B-genome including B1, B2, B3, and B4 subclades (98% PP, 88% BS). Subclade B1 consisted of one *B. juncea*. Subclade B2 consisted of eight *B. juncea*. Subclade B3 contained one *B. nigra* (GQ983033), one *B. carinata*, and two *B. juncea*. Subclade B4 contained one *B. nigra* and 27 *B. juncea* (97% PP, 86% BS). It is inferred from Fig. 1 that sequences from the same genome were better included in a clade, but subspecies from the same genome had a closer genetic relationship, with most varieties inhabiting the same branch.

SplitsTree Analyses

The phylogenetic networks are mainly applied to display complicated reticulations above the species level, the relationships between intraspecific individuals and among populations, and the results of phylogenetic inference of contradicting data sets. SplitsTree analysis was performed to detect reticulate evolution among *Brassica* species and subspecies. The exon and intron data were used to yield a split tree using the split-decomposition method. Four distinct groups (I, II, III, and IV) of *Brassicaceae* were recognized (Fig. 2). Group I included 35 sequences from the B-genome, group II 46 sequences from the A-genome, group III 16 sequences from the C-genome, while six *R. sativus* sequences formed group IV. These classification results were associated with the ML phylogenetic tree result. In terms of network shape and number of subclades, sequences from the B-genome had more differentiation compared with sequences from the A-genome and C-genome. Moreover, Chinese *Brassica* had both tree evolution and reticulate evolution (Fig. 2). Many reticulate evolution events occurred during the evolutionary history of Chinese *Brassica* and its related plants.

Network Analyses

Network analyses were used to reconstruct phylogenetic networks and trees, infer ancestral and potential types, and evolutionary branchings and variants. The exon data set was used to yield MJ network due to the absence of recombination signal in its alignment. In the MJ analysis, a circular network node was a single haplotype, and the size of the node was proportional to the number of individuals with the haplotype. Median vectors refer to unsampled nodes assumed by the MJ network analysis, and the number along a branch represents the mutation site. Use of the MJ network illustrated the genealogical relationship between 95 haplotypes derived from 115 sequences (Fig. 3), and revealed a high level of haplotype diversity. Four distinct haplotypes that were recognized corresponded to the C-genome, A-genome, B-genome, and *R. sativus*. The A-genome haplotypes had 21 distinct mutational steps (at position 33, 54, 108, 183, 204, 294, 300, 336, 339, 348, 428, 429, 483, 567, 663, 666, 681, 795, 867, 1137, and 1140) from the C-genome haplotypes, and six mutational steps (at position 1095, 1.32, 1014, 1011, 987, and 978) distinct from the B-genome haplotypes, indicating that the relationship between the A genome and the B

Fig. 1. Maximum likelihood (ML) tree inferred from *Chs* sequences among *Brassica* species in China.

genome was closer than that of the A and C, and B and C genomes.

The A-genome haplotypes yielded four star-like radiations (I, II, III, and IV). Star-like radiation I included five *B. juncea*, and two *B. juncea* var. *tumida* at the central branching points. Star-like radiation II included three *B. rapa* and 23 *B. juncea*, with five *B. juncea* at the central branching points. Star-like radiation III included one *B. rapa* and 11 *B. juncea*, with four *B. juncea* at the central branching points. Star-like radiation IV included four *B. rapa* and two *B. napus*; no accession was at the central branching point, indicating that *B. rapa* may be the parental donor of the A genome in the tetraploid. B-genome haplotypes yielded only one star-like radiation, including one diploid *B. nigra*; 11 *B. juncea* were at the central branching points. This indicated that *B. nigra* may be the parental donor of the B genome in the tetraploid. As there were numerous *B. juncea* in the star-like radiation sequences, mis-alignment

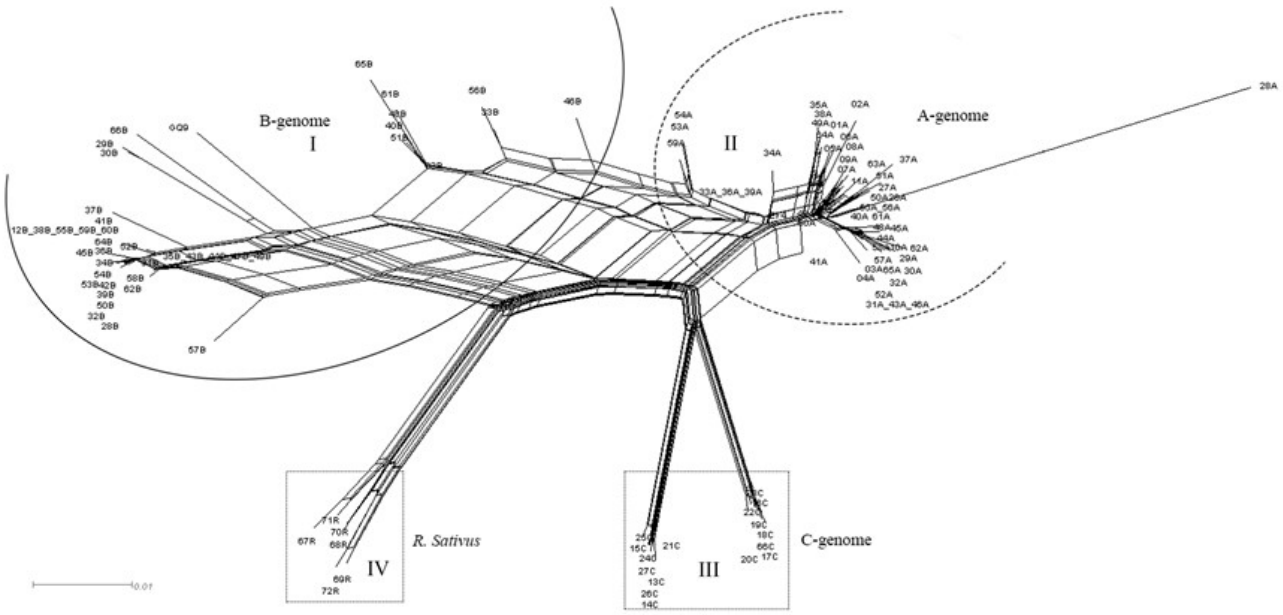

Fig. 2. SplitsTree inferred from *Chs* sequences among *Brassica* species in China.

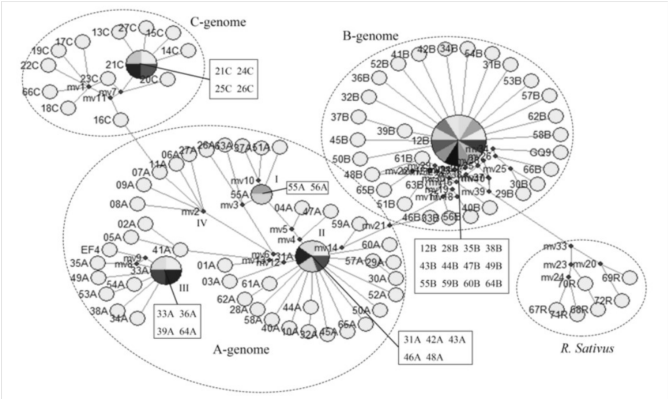

Fig. 3. Median-joining (MJ) network derived from the *Chs* gene sequences among the *Brassica* species in China

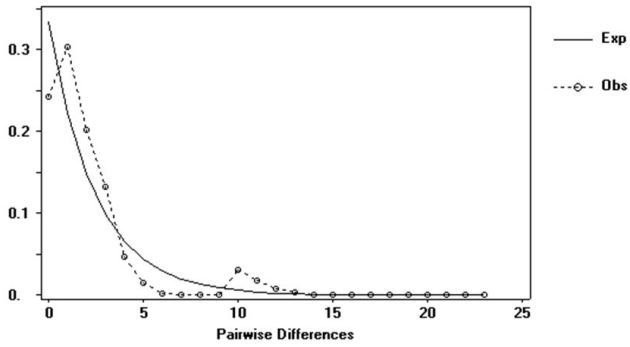

Fig. 4. Mis-alignment analysis in B genome of *Chs* sequences.

analysis was conducted to detect the genetic diversity in the star-like radiation sequences. The results revealed that the genetic diversity of the B-genome in star-like radiation sequences was significantly decreased (Fig. 4).

Genetic Relationships among the A, B, and C Genomes of *Brassica*

Nucleotide variation in the B-polyploid (154 synonymous polymorphisms, 40 non-synonymous polymorphisms) was higher than observed in other taxa (Table 2). As shown by the number of polymorphisms in the A-diploid, A-polyploid, and C-diploid (120, 172, and 25, respectively), the number of polymorphisms in the C-diploid was the lowest. The B-polyploid in *Brassica* retained a high level of variation compared with A-diploid, A-polyploid, and C-diploid genomes on the basis of estimates of π per base pair and θ per base pair. The values of π varied from 0.0143 to 0.0225 and $\hat{\theta}$ from 0.0135 to 0.0313 (Table 3). The highest π and θ were

Table 2. Polymorphic sites of *Chs* gene in the five taxa.

Species	Sample No.	Exon			Intron	
		Syn	Rep	AL	Subs	AL
A-diploid	11	105	15	1177	429	128
A-polyploid	35	141	31	1183	393	87
B-polyploid	33	154	40	1177	431	128
C-diploid	13	6	19	1177	531	228
Radish	6	30	9	1237	543	242

Syn: the number of synonymous polymorphisms in exon; Rep: the number of replacement or nonsynonymous polymorphisms in exons; Subs: the number of base substitutions in intron; AL: the average sequence length of the taxa

Table 3. Sequence polymorphism and neutral evolution test of *Chs* genes in the five taxa.

	N	*H*	*S*	*π*(bp)	*θw*(bp)	D	F_D	F_{fl}	Rm
A-diploid	11	12	121	0.0199	0.0300	-2.2465 (P>0.05)	-2.2465 (P>0.05)	-2.39788 (P>0.05)	8
A-polyploid	35	27	56	0.0173	0.0273	-2.363 (P<0.01)	-4.3648 (0.01<P<0.05)	-4.3733 (0.01<P<0.05)	11
B-polyploid	33	36	156	0.0225	0.0313	-0.9275 (P<0.05)	-1.8906 (P>0.05)	-1.8412 (P>0.05)	16
C-diploid	13	13	69	0.0148	0.0158	1.6095 (P>0.05)	0.4898 (P>0.05)	0.9059 (P>0.05)	1
Radish	6	6	43	0.0143	0.0135	0.3527 (P>0.05)	0.6099 (P>0.05)	0.6081 (P>0.05)	1

N: sample numbers; *S*: the number of segregating sites; *H*: the haplotype numbers; D: Tajima' D; F_D: Fu and Li' D; F_{fl}: Fu and Li' F; Rm: the minimum recombination number

π: Tajima's $\hat{\pi}$ quantifies mean pairwise differences between sequences; *θw*: Watterson's $\hat{\theta}$ refers to an index of the number of polymorphic sites; bp refers to the unit of nucleotide size.

found in B-polyploidy (0.225, 0.313) and the lowest, in the C-diploid (0.0144, 0.0158). The values of θ per base pair in the A-diploid, A-polyploid, and C-diploid were 0.300, 0.0273, and 0.0158, respectively, all of which were larger than observed in the radish out-group (0.0135).

The estimates of Tajima's D, Fu and Li's D, and Fu and Li's F statistics for the A-diploid and the number of C-diploid are positive (Table 3), suggesting that neutral evolution of *Chs* can be accepted for A-diploid and C-diploid genomes. However, the estimates of Fu and Li's D, and Fu and Li's F statistic for the A-polyploid *Chs* gene were significant and large. Furthermore, the Tajima's D statistic for B-polyploidy *Chs* gene was significant, providing evidence for selection of the sequence in A-polyploid and B-polyploid genomes. To further verify factors affecting evolution of the A-polyploid and B-polyploid genomes in the *Chs* gene, HKA tests were performed to detect the inter-group genetic evolution. The HKA test revealed that χ^2 of A-polyploid and B-polyploid genome sequences were 6.086 (P=0.0136) and 2.2674 (P=0.041), respectively, which suggested that artificial selection played an important role in the evolution of Chinese *Brassica*.

Genetic relationships among the five taxa were evaluated based on the number of shared polymorphisms and fixed differences. A shared polymorphism exhibits a history of polymorphism not eliminated by genetic drift. By contrast, a fixed difference suggests that different taxa do not share genetic drift, with independent evolution. Table 4 lists the number of shared polymorphisms and fixed differences between the five genomes. Shared polymorphism values varied from 0 to 63, and fixed differences from 0 to 54. A large number of shared polymorphisms and fewer fixed differences were found between the four genomes of Chinese *Brassica*. On the contrary, *Brassica* and the *R. sativus* out-group shared fewer polymorphisms and exhibited numerous fixed differences, indicating that *R. sativus* was genetically distant from *Brassica*. As shown in Table 4, genetic relationships among the A, B, and C genomes of *Brassica* were close, indicating no, or very recent, divergence between the four genomes of Chinese *Brassica*. This finding revealed that the relationship between A and B genome was closer than that between the A and C, and B and C genomes.

Table 4. The number of shared polymorphisms and fixed differences between taxa.

	A-diploid	A-polyploid	B-polyploid	C-diploid
A-polyploid	63			
	(0)			
B-polyploid	54	61		
	(3)	(10)		
C-diploid	8	7	6	
	(7)	(4)	(19)	
Radish	9	10	10	0
	(22)	(26)	(26)	(54)

Numbers including those enclosed in parenthesis stand for the number of shared polymorphisms and fixed differences, respectively.

DISCUSSION

Sequence Polymorphism of the *Chs* Gene in Chinese *Brassica*

Genetic diversity sequenced by single-copy and low-copy gene sequences can help us better understand the evolution of genes in different populations. In this study, we detected a 1.2-kb domain of the *Chs* gene from 72 individuals, with 113 sequences representing Chinese *Brassica* species and related species. Overall, 272 variable, 996 conserved, 166 informative sites, and 95 singletons were found in the *Chs* sequences. The values of θ per base pair in the A-diploid, A-polyploid, B-polyploid, and C-diploid were 0.0300, 0.0273, 0.0313, and 0.0158, respectively, all of which were larger than the value for the radish out-group (0.0135). As illustrated in the phylogenetic tree,

sequences from all *Brassica* separated into four well-supported clades and 10 subclades. Moreover, the MJ network illustrated the genealogical relationship between 95 haplotypes derived from 115 sequences, thus revealing a high level of haplotype diversity. Consistent with results reported by Wang et al. (2007), Han et al. (2007), Zhao et al. (2009), Yao et al. (2012), Fang et al. (2013), and Liu et al. (2014a), the highest level of genetic diversity was detected in Chinese *Brassica*. This information provides an overview of the genetic diversity in Chinese *Brassica* and can be used to create genetic resources for the management of Chinese *Brassica* breeding programs through distant hybridization of species. Otherwise, as shown in the phylogenetic tree, the diversity among many subspecies was close. It might be that we selected many subspecies with a similar genetic background. Natural hybridizations between *Brassica* members occur frequently, resulting in intermediate or entirely new types with similar genetic relationships (Chen et al. 2013).

Phylogenetic Relationships of Chinese *Brassica*

For many years, relationships within Chinese *Brassica* species and subspecies were the subject of much controversy. Traditionally Chinese *Brassica* were classified into 14 species, 11 subspecies, and one variety, including *B. rapa* var. *rapa* (*B. campestris*), *B. rapa* var. *pekinensis* (*B. pekinensis*), *B. rapa* var. *narinosa* (*B. narinosa*), *B. rapa* var. *parachinensis* (*B. parachinensis*), *B. juncea* var. *megarrhiza* (*B. caulorapa*), and *B. oleracea* var. *napobrassica* (*B. napobrassica*) as individual species (Wang et al. 2006). In agreement with reports by Wang et al. (2006) and Chen et al. (2013), the present *Chs* gene data grouped *B. campestris*, *B. pekinensis*, *B. narinosa*, *B. parachinensis, B. caulorapa*, and *B. napobrassica* species together, with other subspecies scattered into one subclade, indicating that the six species should be classified as within *Brassica*. Based on their study on the characteristics of leaves, petals, seeds, pollen morphology, type of aperture, and sculpture of exine in *Brassica*, Wang et al. (2006) suggested that Chinese *Brassica* should be classified into three groups: *Pekinensis*, *Juncea*, and *Oleracea*. In our *Chs* data, two copies of ancestral allelic *Chs* gene types were successfully cloned in each tetraploid species, while three copies of ancestral allelic *Chs* gene types were obtained in all tetraploids. The copy of the *Chs* gene from the tetraploid and diploid is a well-formed homolog. From our phylogenetic tree, the sequences from all *Brassica* taxa were separated into three well-supported clades in accordance with the A-genome, B-genome, and C-genome revealed by *Chs*. This result reinforces the data presented by Wang et al. (2006), which suggested that Chinese *Brassica* could be divided into three sections: *Pekinensis* (AA genome), *Juncea* (BB, AABB genome) and *Oleracea* (CC, AACC).

Based on inter-simple sequence repeat (ISSR) markers, Du et al. (2009) suggested that in China *B. rapa* can be classified into two groups: *Pakchoi* and *Pekinensis*. In our data, *B. rapa* was included in four subclades: subclade A1 consisted of Chinese cabbage, flowering cabbage, turnip, turnip type rape, and wild rape; subclade A2 consisted of Chinese cabbage and pakchoi; subclade A3 consisted of one flowering cabbage; and subclade A4 contained Chinese cabbage and wild rape. These findings were partly congruent with the results obtained by Du et al. (2009). As shown in Fig. 3, *B. oleracea* includes C1 and C2 subclades: Subclade C1 consisted of eight *B. oleracea* including purple kale, cauliflower, Brussels sprout, and turnip cabbage mustard, which are more evolved; subclade C2 consisted of six *B. oleracea* types including head cabbage, white kale, winter rape, kohlrabi, and rapeseed, which are the original types. The use of SSR markers in *B. oleracea* by Song et al. (2013) presented a similar result. The classification results of *B. juncea* based on *Chs* sequences were not in accordance with the morphological classifications obtained by Li et al. (2014), but are in line with reports by Qi et al. (2007) and Yao et al. (2012) that the traditional phenotypic classification of *B. juncea* is not wholly supported by the molecular results. This may be because of the asymmetrical evolution of polyploid genomes and human selection in *Brassica* (Liu et al. 2014b). As many subspecies of the same genome have a close genetic relationship, the classification of some species and subspecies needs to be further discussed.

SplitsTree analyses indicated that many reticulate evolution events have occurred during the evolutionary history of Chinese *Brassica*. Since there is no reproductive isolation among *Brassica*, species and subspecies may occur as natural hybrids and reticulate evolution events. The mis-alignment analysis revealed that the genetic diversity of B-genome in star-like radiation sequences was significantly decreased (Fig. 4). It was presumed that a large number of natural

mutations occurred in Chinese mustard during a special historical period, then formed different varieties and cultivars. The results of the network analyses further reinforced the results of Kaur et al. (2014) that the original parental species of Chinese *Brassica* are *B. rapa*, *B. nigra*, and *B. oleracea*, in agreement with the triangle of U.

Genetic Relationships among A, B, and C Genomes of *Brassica*

The relationships between the A, B, and C genomes of *Brassica* have received increasing scientific attention. In terms of shared polymorphisms and fixed differences, genetic relationships among A, B, and C genomes are close, indicating no, or very recent, divergence between the three Chinese *Brassica* genomes. This result agrees with the reports by Ge and Li (2007) that the relationship between the A and B genome was closer than that between the A and C, and B and C genomes. However, Li et al. (2014) regarded the relationship between A and C genomes as being closer than that between A and B, and B and C genomes. In terms of the estimates of π per base pair and θ per base pair, the greatest sequence variation was found in the B-polyploid, followed in turn by the A-diploid, A-polyploid, and C-diploid, indicating that the *Chs* sequences of the A-genome, B-genome, and C-genome are evolutionarily distinct. The nucleotide sequence diversity (π) of the B-diploid was higher than that of the A-diploid and the A-polyploid, indicating that the *Chs* sequence of the B-genome may have evolved faster than that of the A-genome. The same result was found when using SplitsTree, with the degrees of differentiation being larger in the B-genome than in the A and C genomes of *Brassica* genera. On the contrary, Liu et al. (2014b) reported that the A-genome and the C-genome evolved faster than the B-genome in *Brassica*. This may be because the accessions and methodology used were different, and the evolution of different genes was different. Artificial selection may have caused the sequence diversity of the A-diploid to be higher than that of the A-polyploid. As *Brassica* are widely cultivated for vegetable and cooking oil use, domestication has had a major effect during the evolution and cultivar creation of Chinese *Brassica*. This study failed to compare the relationship among the diploid B genome and polyploid C genome, owing to the small amounts of *B. nigra*, *B. napa*, and, *B. carinata* material distributed in China.

CONCLUSION

We analyzed the molecular phylogenetic relationships of Chinese *Brassica* species and their closely related genera using data from the single-copy nuclear gene *Chs* sequences. Sequences from *Brassica* were separated into three well-supported groups. This result revealed that Chinese *Brassica* could be divided into three sections – *Pekinensis*, *Juncea*, and *Oleracea*. SplitsTree analysis showed that both tree and reticulate evolution existed, and artificial selection played an important role in the evolution of Chinese *Brassica*. Network analysis suggested that *B. rapa* var. *oleifera*, *B. nigra*, and *B. oleracea* were the parental donors of the A genome, B genome, and C genome in the allotetraploid, respectively. It was presumed that a large number of natural mutations occurred in Chinese *Brassica* during a special historical period. More shared polymorphisms than fixed differences were found among the A, B, and C genomes, indicating that genetic relationships among these genomes of *Brassica* were close. The relationship between the A and B genomes was closer than that between the A and C, and B and C genomes in Chinese *Brassica*.

ACKNOWLEDGMENTS

This report was supported by the Normal Program for Frontiers and Applications Research of CSTC (cstc2016jcyjA0136).

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