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misMM: An Integrated Pipeline for Misassembly Detection Using Genotyping-by-Sequencing and Its Validation with BAC End Library Sequences and Gene Synteny

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As next-generation sequencing technologies have advanced, enormous amounts of whole-genome sequence information in various species have been released. However, it is still difficult to assemble the whole genome precisely, due to inherent limitations of short-read sequencing technologies. In particular, the complexities of plants are incomparable to those of microorganisms or animals because of whole-genome duplications, repeat insertions, and Numt insertions, etc. In this study, we describe a new method for detecting misassembly sequence regions of *Brassica rapa* with genotyping-by-sequencing, followed by MadMapper clustering. The misassembly candidate regions were cross-checked with BAC clone paired-ends library sequences that have been mapped to the reference genome. The results were further verified with gene synteny relations between *Brassica rapa* and *Arabidopsis thaliana*. We conclude that this method will help detect misassembly regions and be applicable to incompletely assembled reference genomes from a variety of species.

Keywords: BAC end library, gene synteny, genotyping-by-sequencing, miassembly, next-generation sequencing, reference genome

Introduction

The genomics era has opened in earnest with the completion of the Human Genome Project. With the development of next-generation sequencing (NGS) technologies, the amount of genomics data has exploded, and sequencing targets have become very diverse. As of 2017, there are 7,930 species of eukaryotes, 192,677 species of bacteria, and 1,412 species of archaea that have been officially registered in NCBI. As the Nagoya Protocol is initiated, it is expected that these numbers will continue to increase in the future due to the policies of each country to secure information on biological genetic resources [1, 2]. Despite the fact that the cost of genomic analysis is declining, there are still a number of technical problems that make it difficult to sequence the genome completely [3]. For example, misa-

ssembly due to the inherent limitations of NGS technology is well known [4-6]. Especially in plants, there are many barriers that make plant genomes hard to sequencing, such as Numts, repeats, and genome duplication events [7-9].

Genotyping-by-sequencing (GBS) is a technology that allows high-throughput genotyping by applying NGS technology. It is used to analyze single nucleotide polymorphisms (SNPs) in populations to find molecular markers that are related to phenotype and genotype or to draw genetic linkage maps for plant breeding. By analyzing the pattern of GBS data along each chromosome, one can find out where the gene crossover occurs. On the other hand, a small block that interrupts an otherwise continuous GBS pattern is genetically non-ideal and implies a misassembled region. Therefore, we explored the application of GBS in the detection of misassemblies [10-12].

Brassicaceae is a mustard family containing 372 genera

and 4,060 accepted species, and its varieties are cultivated as economically valuable crops not only in East Asia but also globally [13]. The triangle of U theory states that the differentiation of an allotetraploid of *Brassica* species—*Brassica juncea* (AABB), *Brassica napus* (AACC), and *Brassica carinata* (BBCC)—occurs due to the polyploidization of diploid *Brassica* species: *Brassica rapa* (AA), *Brassica nigra* (BB), and *Brassica oleracea* (CC). This theory has been proven by genomic analysis by NGS of *Brassica* species [14-25]. Research on the correlation between the genetic information and the nutrient content of crops has been actively conducted in *Brassica* genomes [26]. The recently published *B. rapa* V2.1 genome sequence shows much improved quality, as well as a number of misassembly corrections over the previous version, V1.5 [17]. This offers an interesting opportunity to test the potential of misassembly detection, based on GBS data.

In this study, we propose a user-friendly pipeline, called misMM, which automatically identifies misassembled candidate blocks (MCBs) and adjacent to destination blocks (ADBs) and plots the genetic map of MCBs by using raw GBS data sorted by MadMapper [27]. These results are verified by using the BAC end-sequence library published in NCBI and the gene synteny relation between *Arabidopsis thaliana* and *B. rapa* [28-31].

Methods

Data source

The end sequences of *B. rapa* accession Chiifu-401-42, a Chinese cabbage BAC library (KBrH, KBrB, and KBrS), were downloaded from NCBI and used to verify the putative misassembly genome regions. In order to investigate the gene synteny relation between *A. thaliana* TAIR10 and *B. rapa* genome V1.5, the corresponding general feature format

(GFF) annotation files and protein sequences of each species were downloaded from http://ensemblgenomes.org and http://brassicadb.org, respectively. The GBS data were produced by a previous study that investigated the correlation between flavonoid content and the genotype of *B. rapa* in 69 individuals of a doubled haploid F2 generation obtained by microbial culture of an F1 generation cross of two subspecies—yellow sarson of LP08 (*B. rapa* ssp. *tricolaris*) and pak choi of LP21 (*B. rapa* ssp. *chinensis*)—with distinct morphologies [26]. From the study, genotype data were obtained at a total of 8,176 positions.

Configuration of the misMM pipeline for misassembled block detection

misMM, a pipeline for genome misassembled block detection, was written in a Linux shell and with Python ver. 2.7 in-house codes. The first step is preprocessing: after loading all GBS raw data files, markers with a missing value of over 8% were filtered out. If the neighboring positions had the similar GBS pattern with consistency, they were grouped into one block. Our script then automatically prepared the three kinds of input files (.loc, IDs, and maps) for Mad-Mapper (UC Davis) [27], a package that specializes in recombinant inbred lines analysis using large genetic markers and easy visualizes the 2D pairwise matrix. The next step is the linkage grouping and block shuffling step, performed with MadMapper. By using the default parameters of MadMapper_RECBIT (rec_cut, 0.2; bit_cut, 100; data_cut, 25; allele_dist, 0.33; missing_data, 50; trio_analysis, TRIO; double_cross, 3), linkage grouping and marker extraction were performed by generating a pairwise matrix between GBS patterns of each block. Subsequently, block shuffling was performed by MadMapper_XDELTA (marker fixation, FIXED; shuffle option, SHUFFLE; shuffle block, 6; shuffle step, 3) with each clustered block. At the end of this
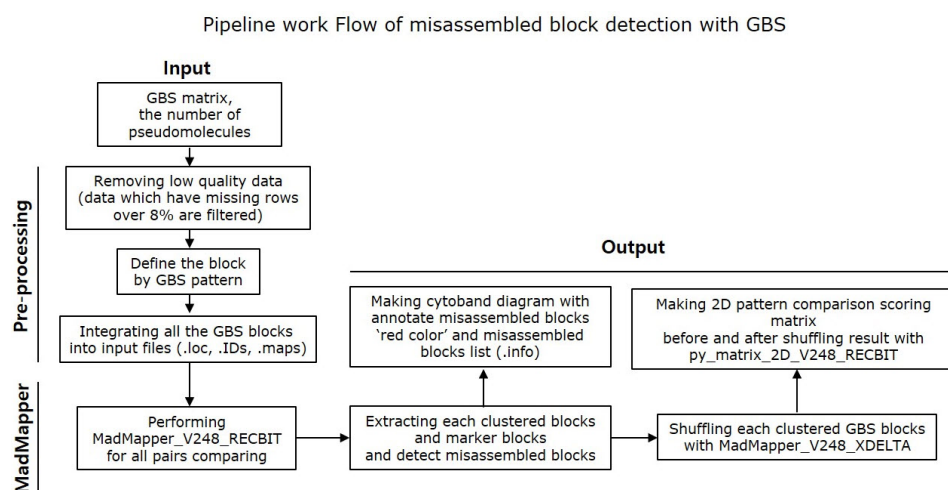

Fig. 1. Total work flow of misMM. GBS, genotyping-by-sequencing.

process, it plotted a genetic map diagram with putative misassembled blocks. In addition, it also generated 2D heatmap graphs for comparing before and after the block shuffling. All of the work flow of this pipeline is described in Fig. 1. The misMM pipeline scripts can be downloaded from http://sskimbnas.ipdisk.co.kr:80/publist/HDD1/misMM/misMM.tar.gz.

Validation using BAC end sequences

In order to confirm the misassembled blocks with experimental data, we extracted 41,969 pairs of end sequences from the BAC libraries (KBrS, KBrH, and KBrB) of *B. rapa* and carried out sequence alignment against the *B. rapa* reference genome sequence using Nucmer (MUMmer3.23) with the proper options (--maxmatch, use all anchor matches; -g, global alignment; -I, >95%; -r, sort output lines by reference). The Nucmer results were then filtered for discordant BAC end pairs with one end aligned to the MCB and the other end to the ADB.

Validation using gene synteny relation between *A. thaliana* and *B. rapa*

For validation with gene synteny, the protein sequence of *B. rapa* were matched to those of *A. thaliana* using BLASTP (Blast 2.2.26), and the top four hits for each query were retained. The tabulated results were then sorted, based on the genomic coordinates of each protein, and the gene synteny relation was examined manually.

Results and Discussion

misMM was developed to provide a streamlined and yet simple-to-use pipeline for the detection of misassembled regions, so-called MCBs, based on GBS data (Fig. 1). This pipeline was tested with the GBS data of *B. rapa* against the *B. rapa* V1.5 reference genome, which is known to have some misassembled regions compared to the recently published V2.1 genome [17]. The original linkage score heatmap that was produced by MadMapper showed many off-diagonal cells with a low score that were often clustered in stretch (Fig. 2 left panel). The off-diagonal blocks scoring less than 0.33 were defined as MCBs (Table 1, Fig. 3). For each MCB, the corresponding ADB was identified by MadMapper, based on the linkage score (Table 1). The subsequent shuffled heatmap showed clean clustering, with no low-scoring off-diagonal blocks, implying the unambiguousness of the GBS pattern in detecting misassemblies (Fig. 2 right panel). The MCBs and ADBs were distributed throughout the entire pseudomolecule. A total of 16 MCBs had an average block size of 65,477 bp, and the largest one was 410,190 bp. The average size of the ADBs was 746,707 bp, with a maximum of 4,936,893 bp. The fact that only a few small MCBs were detected and that the corresponding ADBs were large in size implies that the *B. rapa* V1.5 genome is well assembled overall but has a few problematic regions, as shown by the recent update of the genome [17].

We used two sets of data to validate that the ADBs were indeed in the neighboring area of the MCBs. The first one was used to find discordant BAC end pairs with one end
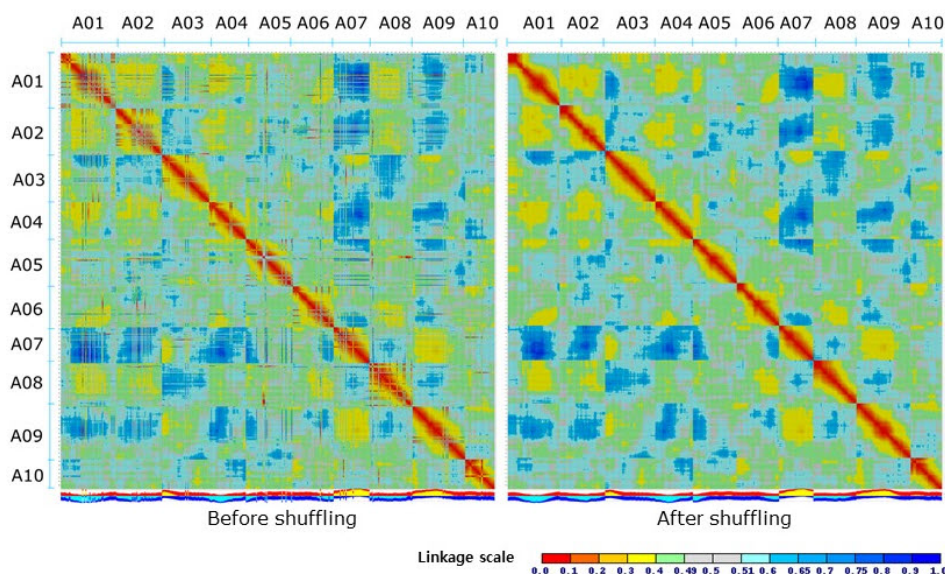

Fig. 2. Before and after the results of the 2D matrix graphs of the MadMapper block shuffling analysis. A01 through A10 indicate the *Brassica rapa* pseudomolecules.

Table 1. Results of misassembled block detection analysis in *Brassica rapa* with misMM

Block No.	Misassembled candidate block				Adjacent to destination block				Synteny relation	Count of BAC end
	Chr No.	Start position	End position	Block size (bp)	Chr No.	Start position	End position	Block size (bp)		
1	A01	10,335,503	10,336,457	955	A07	2,718,763	2,760,427	41,665	No gene	1
						2,970,361	3,340,395	370,035		
						4,284,326	5,685,009	1,400,684		
						5,782,516	8,114,350	2,331,835		
						8,306,623	8,390,460	83,838		
						8,462,236	9,063,378	601,143		
2	A01	11,453,104	11,488,558	35,455	A04	3,271,457	4,978,203	1,706,747	Related	6
						5,227,803	6,734,498	1,506,696		
						7,605,871	7,605,928	58		
3	A01	11,830,981	-	1	A05	10,274,396	14,490,617	4,216,222	Related	1
	A07	13,576,261	-	1		14,602,065	14,704,957	102,893		
	A08	1,389,252	1,419,543	30,292		14,946,890	15,735,698	788,809		
						6,968,479	7,090,412	121,934		
						7,231,217	7,782,948	551,732		
						7,825,594	8,040,473	214,880		
						8,683,679	9,511,317	827,639		
4	A01	17,853,386	17,853,417	32	A03	28,233,583	28,599,515	365,933	Related	2
	A01	21,422,470	21,756,693	334,224		28,622,787	29,191,693	568,907		
	A02	26,385,973	26,386,023	51		29,806,067	31,527,446	1,721,380		
5	A01	23,266,604	23,424,555	157,952	A06	10,280,840	10,357,155	76,316	Related	13
	A02	13,440,136	13,440,137	2		10,732,633	14,236,176	3,503,544		
	A02	21,066,162	21,066,274	113		14,450,457	14,559,524	109,068		
						8,950,753	10,162,388	1,211,636		
6	A01	8,706,169	8,950,670	244,502	A09	11,293,419	11,528,445	235,027	Related	63
	A06	19,457,789	19,703,630	245,842		11,610,344	14,668,929	3,058,586		
						14,915,372	15,794,376	879,005		
						15,949,568	18,361,629	2,412,062		
						19,064,188	22,487,944	3,423,757		
						22,634,782	23,337,316	702,535		
						23,489,828	23,489,836	9		
7	A02	21,427,161	-	1	A10	11,579,416	-	1	No gene	0
						176,000	1,638,829	1,462,830		
						1,765,780	1,766,618	839		
						1,786,668	1,792,156	5,489		
						3,686,351	5,224,789	1,538,439		
						5,335,436	5,459,255	123,820		
						5,648,752	5,693,352	44,601		
8	A03	15,343,238	-	1	A08	2,368,697	3,803,367	1,434,671	Related	2
						4,037,929	4,357,798	319,870		
						4,787,505	4,835,708	48,204		
						5,188,553	6,046,948	858,396		
						7,117,019	7,501,164	384,146		
						7,559,836	8,722,062	1,162,227		
						9,000,508	9,002,057	1,550		
9	A05	8,144,773	8,162,600	17,828	A08	19,141,593	19,320,715	179,123	Related	16
		8,217,883	8,234,265	16,383		19,394,784	19,497,679	102,896		
		8,250,451	8,352,384	101,934		19,568,112	19,621,358	53,247		
						19,674,213	19,711,491	37,279		

Table 1. Continued

Block No.	Misassembled candidate block				Adjacent to destination block				Synteny relation	Count of BAC end
	Chr No.	Start position	End position	Block size (bp)	Chr No.	Start position	End position	Block size (bp)		
10	A05	9,669,449	10,079,638	410,190	A01	10,376,926	10,494,252	117,327	Related	23
						10,687,155	11,394,090	706,936		
						11,519,211	11,744,579	225,369		
						11,900,084	16,836,976	4,936,893		
						16,848,291	17,125,316	277,026		
						17,226,336	17,789,202	562,867		
						17,860,877	18,575,071	714,195		
						9,305,241	9,707,259	402,019		
						9,711,107	10,267,937	556,831		
11	A07	2,319,220	2,321,114	1,895	A01	24,324,484	24,353,432	28,949	Related	0
						24,402,955	24,488,344	85,390		
						24,619,832	24,806,034	186,203		
						24,920,288	24,920,419	132		
12	A07	3,920,950	4,009,069	88,120	A10	11,579,416		1	Related	0
	A08	3,927,665	-	1		176,000	1,638,829	1,462,830		
						1,765,780	1,766,618	839		
						1,786,668	1,792,156	5,489		
						3,686,351	5,224,789	1,538,439		
						5,335,436	5,459,255	123,820		
						5,648,752	5,693,352	44,601		
13	A07	8,271,542	8,274,604	3,063	A03	12,032,914	12,032,953	40	Related	5
						12,049,203	12,406,487	357,285		
						12,473,776	13,917,498	1,443,723		
						14,019,224	14,200,642	181,419		
						14,222,379	14,355,939	133,561		
14	A08	11,266,789	-	1	A03	25,996,840	26,033,638	36,799	Related	0
						26,067,147	27,037,677	970,531		
						27,139,966	27,943,662	803,697		
15	A05	8,552,907	8,593,005	40,099	A02	11,596,619	13,185,910	1,589,292	Related	0
	A08	1,584,456	1,594,851	10,396		13,498,575	14,449,253	950,679		
						14,804,284	18,303,089	3,498,806		
						18,558,399	19,378,431	820,033		
						19,548,342	19,666,145	117,804		
						19,787,176		1		
16	A08	4,941,300	4,969,852	28,553	A10	11,146,660	11,437,447	290,788	Related	0
						11,664,229		1		
						11,764,627	11,814,368	49,742		
						11,928,253	12,032,475	104,223		

aligned to the MCB and the other end aligned to the ADB. For example, the MCB of block number 2 in Table 1 was located in pseudomolecule A01, ranging from 11,453,104 to 11,488,588, while its corresponding ADBs were found in A04. Table 2 shows the mapping results of the six BAC end pairs of this block, the sizes of which ranged from 671 bp to 1,000 bp, with a mapping identity higher than 97.93%. While one end of the BAC pairs was mapped to the corresponding MCB in A01, all of the other ends were mapped within the ADB, ranging from 3,271,457 to 4,978,203 in A04. Likewise, 10 out of 16 blocks listed in Table 1 could be confirmed by the BAC end results. The true locations of these blocks could be estimated within the span of the corresponding BAC (average 110 kbp). The rest could not be confirmed, probably due to the distance between the MCB and ADB, making it incompatible with the BAC size.

The other validation method was the use of the gene synteny relation. Compared to the *A. thaliana* genome, there

is evidence that the *B. rapa* genome has undergone triplication [32]. Accordingly, most of the *A. thaliana* genes are preserved in gene synteny blocks at three different places. Within block number 2 in Table 1, two *B. rapa* genes are annotated: Bra033489 and Bra033490 (Table 3). For all 16 genes flanking these two genes, orthologs were identified by BLASTP (Table 4). Eight *A. thaliana* genes in the middle—including the orthologs of two genes, AT4G14330 and AT4G14350—were out of order and broke the continuity of the synteny in the region. This is consistent with our finding that this MCB is truly misplaced in *B. rapa* genome V1.5. The true locations of the two *B. rapa* genes in this MCB can be inferred by mapping the flanking genes of AT4G14330 and AT4G14350 to the *B. rapa* genome (Table 5). Indeed, a total of six *A. thaliana* flanking genes were mapped to the *B. rapa*

orthologs that were found in the corresponding ADBs. As expected, the gene synteny of this region is also well preserved. In this way, we can estimate the approximate relative locations of these two genes. Based on this relationship, an analysis was carried out with regard to the relationship of the protein orthologs and gene coordination between the two species. First, two genes were annotated in an example block (Table 3). When these two genes were found in a table arranged by the coordinates of the *B. rapa* gene, there was no continuity between the ortholog genes and the surrounding genes (Table 4). But, when we sorted this based on the coordination of *A. thaliana*, the ortholog genes belonging to the ADB were located consecutively around the gene belonging to the MCB (Table 5). Furthermore, the gene order that was inferred here was confirmed in the updated *B. rapa* V2.1 genome that was recently published [17].

In recent years, studies of expression quantitative trait loci that affect mRNA expression or protein expression using SNPs and studies to find markers that affect the environmental adaptation of plants have been becoming widely embraced [33]. For such works, accurate reference genome assembly is required. Toward that goal, our misMM pipeline is a useful tool for the identification of misassemblies in complex genomes using GBS data.
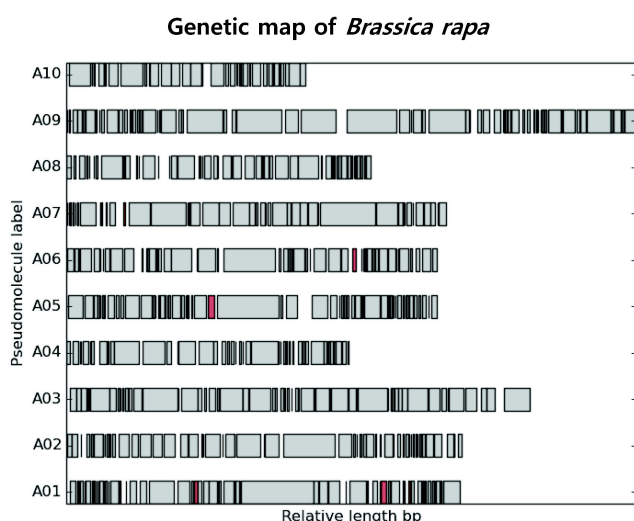
Genetic map of *Brassica rapa*

Fig. 3. Example of *Brassica rapa* genetic map made with misMM pipeline. Red colors indicate misassembled candidate blocks.

Table 3. Information on genes included in example misassembled candidate block

Chr No.	Type	Start point	End point	*Brassica rapa* ID
A01	Gene	11,455,026	11,470,735	Bra033489
A01	Gene	11,451,545	11,454,600	Bra033490

Table 2. Example of validation of BAC end library results

BAC ends library ID	gi No.	Length (bp)	Identity (%)	*Brassica rapa*		
				Chr No.	Start position	End position
KBrB037L22F	84732862	671	97.93	A01	11,474,904	11,475,144
KBrB037L22R	84732863	671	99.4	A04	4,869,416	4,870,085
KBrB039C19R	84733951	869	99.65	A01	11,471,320	11,472,188
KBrB039C19F	84733950	822	99.76	A04	4,884,036	4,884,855
KBrB043O24F	84737591	874	99.89	A01	11,452,951	11,453,822
KBrB043O24R	84737592	816	100	A04	4,884,025	4,884,840
KBrB077H15F	84762968	617	98.92	A01	11,474,904	11,475,088
KBrB077H15R	84762969	646	100	A04	4,884,386	4,885,031
KBrB097P17F	114827207	1,000	98.2	A01	11,471,303	11,472,294
KBrB097P17R	114827208	937	98.16	A04	4,883,252	4,884,169
KBrH087A11R	84341421	831	99.88	A01	11,466,761	11,467,587
KBrH087A11F	84341072	844	99.63	A04	4,977,838	4,978,643

Table 4. Example of protein ortholog list, sorted by *Brassica rapa* gene coordination

	Brassica rapa				Arabidopsis thaliana				Comments
ID	Chr No.	Start position	End position	ID	Chr No.	Start position	End position		
Bra033497	A01	11,382,249	11,386,827	AT4G15570	Chr4	8,892,607	8,898,999		-
Bra033496	A01	11,388,925	11,390,027	AT4G15563	Chr4	8,890,879	8,892,526		-
Bra033495	A01	11,393,659	11,396,663	AT4G15560	Chr4	8,883,907	8,887,565		-
Bra033494	A01	11,410,610	11,412,043	AT4G15550	Chr4	8,877,590	8,879,327		-
Bra033493	A01	11,412,702	11,414,443	AT4G15545	Chr4	8,875,918	8,877,799		-
Bra033492	A01	11,445,862	11,446,743	AT5G49420	Chr5	20,034,674	20,036,170		-
Bra033491	A01	11,450,091	11,451,172	AT4G14320	Chr4	8,241,732	8,243,910		-
Bra033490	A01	11,451,545	11,454,600	AT4G14330	Chr4	8,244,194	8,247,444		Misassembled candidate
Bra033489	A01	11,455,026	11,470,735	AT4G14350	Chr4	8,256,086	8,260,787		Misassembled candidate
Bra039534	A01	11,504,946	11,505,630	AT2G35280	Chr2	14,859,378	14,860,200		-
Bra039535	A01	11,504,946	11,505,422	AT2G35280	Chr2	14,859,378	14,860,200		-
Bra039536	A01	11,504,994	11,505,630	AT2G35280	Chr2	14,859,378	14,860,200		-
Bra039538	A01	11,510,855	11,512,648	AT3G59380	Chr3	21,944,178	21,945,943		-
Bra039539	A01	11,514,776	11,515,144	AT4G15530	Chr4	8,864,828	8,870,967		-
Bra039540	A01	11,516,583	11,521,200	AT4G15530	Chr4	8,864,828	8,870,967		-
Bra039541	A01	11,521,728	11,523,067	AT4G15520	Chr4	8,862,815	8,864,618		-

Table 5. Example of protein ortholog list, sorted by *Arabidopsis thaliana* gene coordination

	Brassica rapa				Arabidopsis thaliana			Comments
ID	Chr No.	Start position	End position	ID	Chr No.	Start position	End position	
Bra032781	A04	4,968,584	4,971,945	AT4G14290	Chr4	8,225,481	8,230,281	Included in ADB
Bra032782	A04	4,962,259	4,963,651	AT4G14305	Chr4	8,235,093	8,236,715	Included in ADB
Bra033490	A01	11,451,545	11,454,600	AT4G14330	Chr4	8,244,194	8,247,444	Included in MCB
Bra033489	A01	11,455,026	11,470,735	AT4G14350	Chr4	8,256,086	8,260,787	Included in MCB
Bra033487	A04	4,917,814	4,918,407	AT4G14380	Chr4	8,285,766	8,286,772	Included in ADB
Bra033486	A04	4,915,949	4,917,119	AT4G14385	Chr4	8,286,986	8,288,800	Included in ADB
Bra033483	A04	4,882,642	4,883,358	AT4G14440	Chr4	8,306,745	8,307,753	Included in ADB
Bra033482	A04	4,873,477	4,873,797	AT4G14450	Chr4	8,309,474	8,310,058	Included in ADB

Alternative alignments due to genome triplication have been removed.
ADB, adjacent to destination block; MCB, misassembled candidate blocks.

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Authors' contribution

Conceptualization: YJK, JSK
Data curation: YJK
Formal analysis: YJK
Funding acquisition: SK
Methodology: YJK
Writing – original draft: YJK
Writing – review & editing: SK, JSK

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