ISSN 1226-8763
Kor. J. Hort. Sci. Technol. 32(4):535-543, 2014 http://dx.doi.org/10.7235/hort.2014.14109

Research Report

Single Nucleotide Polymorphism Marker Discovery from Transcriptome Sequencing for Marker-assisted Backcrossing in *Capsicum*

Jin-Ho Kang[1,2,3†], Hee-Bum Yang[1,2,3†], Hyeon-Seok Jeong[1,2,3], Phillip Choe[1,2,3], Jin-Kyung Kwon[1,2,3], and Byoung-Cheorl Kang[1,2,3*]

[1]*Department of Plant Science, Seoul National University, Seoul 151-921, Korea*
[2]*Vegetable Breeding Research Center, Seoul National University, Seoul 151-921, Korea*
[3]*Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea*

Abstract: Backcross breeding is the method most commonly used to introgress new traits into elite lines. Conventional backcross breeding requires at least 4-5 generations to recover the genomic background of the recurrent parent. Marker-assisted backcrossing (MABC) represents a new breeding approach that can substantially reduce breeding time and cost. For successful MABC, highly polymorphic markers with known positions in each chromosome are essential. Single nucleotide polymorphism (SNP) markers have many advantages over other marker systems for MABC due to their high abundance and amenability to genotyping automation. To facilitate MABC in hot pepper (*Capsicum annuum*), we utilized expressed sequence tags (ESTs) to develop SNP markers in this study. For SNP identification, we used Bukang F_1-hybrid pepper ESTs to prepare a reference sequence through de novo assembly. We performed large-scale transcriptome sequencing of eight accessions using the Illumina Genome Analyzer (IGA) IIx platform by Solexa, which generated small sequence fragments of about 90-100 bp. By aligning each contig to the reference sequence, 58,151 SNPs were identified. After filtering for polymorphism, segregation ratio, and lack of proximity to other SNPS or exon/intron boundaries, a total of 1,910 putative SNPs were chosen and positioned to a pepper linkage map. We further selected 412 SNPs evenly distributed on each chromosome and primers were designed for high throughput SNP assays and tested using a genetic diversity panel of 27 *Capsicum* accessions. The SNP markers clearly distinguished each accession. These results suggest that the SNP marker set developed in this study will be valuable for MABC, genetic mapping, and comparative genome analysis.

Additional key words: expressed sequence tag, linkage map, marker-assisted breeding

Introduction

Hot pepper (*Capsicum annuum*) is a crop of major economic importance that is commercially cultivated in China, Korea, the East Indies, and the United States of America, among many other countries (Shao et al., 2008). Worldwide production of hot peppers has been estimated to be 14-15 million tons a year (Weiss, 2002). In fact, hot pepper is the vegetable accounting for the largest planting area in Korea.

In commercial breeding, new traits are commonly introduced into elite breeding lines using conventional backcross methods, which involve time consuming efforts to transfer target genes into the genetic background of a recipient parent. Recently marker-assisted backcrossing (MABC), which is more efficient and faster than conventional backcrossing, has been generally accepted as an advanced plant breeding technique (Blum et al., 2002). For successful application

*Corresponding author: bk54@snu.ac.kr

[†]These authors contributed equally to this work.

※ Received 17 June 2014; Revised 7 July 2014; Accepted 8 July 2014. This work was supported by a grant from the Next-GenerationBioGreen 21 Program [The Agricultural Genome Center (No. PJ009083) and Plant Molecular Breeding Center (No. PJ00906501201401)], Rural Development Administration, Republic of Korea. The online version of this article (doi:http://dx.doi.org/10.7235/hort.2014.14109) contains supplementary material, which is available to authorized users.

of MABC, factors to consider include the number of target genes to be transferred, the genome size of the crop species, and the availability of a highly saturated map. Among those factors, the availability of highly polymorphic markers with known positions in each chromosome is critical (Varshney et al., 2009). Although molecular markers based on restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and random amplified polymorphic DNA (RAPD) have been developed and used in practical plant breeding (Imelfort et al., 2009; Jung et al., 2010; Kang et al., 2001; Paran et al., 2004; Yoo et al., 2003), such markers still have limitations for use in MABC due to the difficulty of finding polymorphisms among breeding lines and of high throughput genotyping. Recently, single nucleotide polymorphism (SNP) markers have captured attention because of their potential for high-throughput detection and computerization with automated platforms (Jung et al., 2010; Vignal et al., 2002; Yi et al., 2006).

SNPs are single-base differences in DNA between accessions. In plants, SNPs generally occur in populations once every few hundred base pairs (Metzker, 2005). SNP markers can be developed through several methods. For example, SNPs can be identified by simply comparing a candidate sequence to a reference sequence (Nicolai et al., 2012), by whole genome sequencing (WGS; Goff et al., 2002; The Arabidopsis Genome Initiative, 2000), or by sequence alignment of expressed sequence tags (ESTs) to a reference sequence (Jones, 2009; Kota et al., 2001; Labate and Baldo, 2005). For the crops like hot pepper, in which the genome is huge, ESTs have been adopted as an alternative to WGS and as a substrate for cDNA array-based expression analyses (Kim et al., 2008; Rudd, 2003). ESTs are a few hundred base pairs of sequence derived from randomly selected cDNA clones prepared from specific tissues, and EST sequencing is inexpensive compared to WGS. These characteristics led us to test whether SNP markers could be developed from several pepper accessions using ESTs generated with next generation sequencing (NGS) technology.

NGS is a fast and low cost method for the large-scale generation of reliable and robust transcript sequences and identifying and characterizing genetic polymorphisms in plants (Imelfort et al., 2009; Metzker, 2010). The Illumina Genome Analyzer (IGA) used to be the most widely used platform based on amplified sequencing features generated by bridge PCR (Shendure and Ji, 2008). Since the IGA reads only a short sequences (75-100 base pairs; Flicek and Birney, 2009), SNPs can be identified by either de novo assembly of short sequence reads or alignment to the reference. Several factors can interfere with correct sequence alignment; these include missing calls of overlapping genotypes (Anney et al., 2008), false discovery of polymorphic SNPs (Pettersson et al., 2008), homozygote to heterozygote miscalls (Teo et al., 2007), and allelic dropout (Pompanon et al., 2005).

Here, we describe a process for SNP development from transcriptome sequencing of peppers. The resulting SNP primers clearly distinguished between 27 tested *Capsicum* cultivars, demonstrating that the SNP markers developed in this study will be useful resources to facilitate MABC in pepper.

Materials and Methods

Plant Material Preparation for Eight Accessions

Jeju (*Capsicum annuum*), LAM32 (*C. annuum*), Tean (*C. annuum*), CM334 (*C. annuum*), SNU-001 (*C. chinense*), Yuwolcho (*C. annuum*), PI201234 (*C. annuum*) and YCM334 (*C. annuum*) were grown in a growth chamber with 12 h light at 25°C and 12 h dark at 18°C. Leaf tissues at the same stage from the eight accessions were collected. Total RNA was isolated from leaves of each accession with Trizol extraction buffer (Ambion, Carlsbad, CA, USA) as described in the manufacturer's protocol and used for sequencing of transcriptomes.

Sequencing of Transcriptomes for Eight Accessions

Jeju, LAM32 and Tean were sequenced via GAIIx sequencing with 116-bp single-end reads at the National Instrumentation Center for Environmental Management (NICEM). CM334 sequencing data of 101-bp paired-end reads were provided by Dr. Choi from the Plant Genomics Laboratory at Seoul National University. SNU-001, Yuwolcho, PI201234 and YCM334 were sequenced with the GAIIx sequencing platform using the 90-bp paired-end read sequencing method at Beijing Genome Institute (BGI).

Quality Trimming of Reads

The sequence data from each accession were trimmed to reduce the quality deterioration in the 3'-end and 5'-end regions, which negatively affects mapping and assembly. Sickle version 1.0 (https://github.com/najoshi/sickle) was used for quality trimming.

Reference Sequence Preparation

The reference sequence, assembled by commercially available CLC Genomics Workbench software, comprised 31,196 contigs from EST sequences that were mainly derived from Korean F1 hybrid line Bukang, for which data are available at the Korea Research Institute of Bioscience & Biotechnology (KRIBB) (Ashrafi et al., 2012; Kim et al., 2008).

Repeat Masking on the Reference Sequence

Repeat masking was performed on the reference sequence using the 727 repeat sequence library set up by the Plant Genomics Laboratory at Seoul National University because of the presence in *Capsicum* of areas highly abundant in repeat sequence (Yi et al., 2006). The data were collected by the RMBlast program, which is mainly used in NCBI for finding matches between two sequences and attempting to start alignments from these matched places (Johnson et al., 2008). RepeatMasker from the Institute for Systems Biology was used to screen DNA sequences for repeats and areas of low complexity.

Alignment to the Reference Sequence

Sequence alignments to the reference sequence were performed to find SNPs (McPherson, 2009) using the Burrows-Wheeler Transform algorithm from Burrows-Wheeler Aligner version 0.5.9rc1, which requires little memory and so is suitable for reducing the analysis time (Li and Durbin, 2009).

SNP Calling and Filtering

SNP data were collected using SAMtools software and an in-house python script with strict criteria (Li et al., 2009). SNPs from sequencing depth of 25x were filtered in the following order: 1) diallelic SNPs, 2) SNPs found in more than six accessions, 3) SNPs with high PIC value, 4) SNPs for Fluidigm probe design (at least 60 bp from any intron-exon junction or another SNP).

Linkage Mapping

A genetic map was drawn by connecting candidate SNPs after the filtering process to a hybrid of *Capsicum frutescens* cv. *BG2814-6* x *Capsicum annuum* cv. NuMexRNaky RIL population (119 RILs) built at the University of California, Davis (https://pepchip.genomecenter.ucdavis.edu/).

Polymorphism Survey and Genetic Diversity

SNPs positioned in the linkage map were used to design locus-specific markers for the Fluidigm$^{®}$ EP1^{TM} genotyping system. To validate their polymorphism and study the application of the SNP markers, 24 different *C. annuum* accessionss (CM334, YCM334, Tean, Yuwolcho, ECW, Bukang A line, Bukang C line, Lam32, Jeju, Dempsey, DRB, Perennial, 9093, ECW30R, Takanotsume, 35001, 35009, RS202, RS203, RS205, VK-515R, VK515S, LongSweet, AC2212) and three different *C. chinense* accessions (PI159236, Habanero, SNU11-001) were employed. Genomic DNAs of each accessions were extracted by the hexadecyl trimethyl ammonium bromide (CTAB) method from young leaf tissues (Yang et al., 2012). Phylogenetic analysis was carried out using the neighbor-joining method in Darwin5 software (http://darwin.cirad.fr/darwin, Perrier et al., 2003). Tree construction was based on the unweighted neighbor-joining method, and bootstraps were determined from 1000 replicates.

Results

Sequencing of Eight Hot Pepper Accessions and Quality Trimming

We produced a total of 4.1-4.4 Gb with 36-38 million

Table 1. Sequencing information from eight pepper accessions.

Cultivar	Origin	Raw reads			Trimmed reads		
		Bases	Reads	Read length (bp)	Bases	Reads	Read length (bp)
Jeju	Korea	4,341,320,416	37,425,176	116	3,690,172,704	36,990,461	99.8
LAM32	India	4,120,993,728	35,525,808	116	3,596,017,756	35,239,208	102
Tean	Korea	4,356,564,208	37,556,588	116	3,708,990,466	37,165,176	99.8
CM334	Mexico	7,111,852,582	70,414,382	101	5,297,351,327	66,591,676	79.5
SNU-001	Venezuela	3,575,010,600	39,722,340	90	3,440,874,878	39,338,990	87.5
Yuwolcho	Korea	3,579,091,560	39,767,684	90	3,438,315,513	39,381,505	87.3
PI201234	Germplasm in USA	3,575,048,220	39,722,758	90	3,493,467,360	39,637,637	88.1
YCM334[z]	Taiwan	3,525,119,100	39,167,990	90	3,444,541,024	39,081,800	88.1

[z] RIL line derived from a cross between Yolo wonder and CM334 in INRA (France).

reads from Jeju, LAM32 and Tean; a total of 7.1 Gb with 70 million reads from CM334; and a total of 3.5-3.6 Gb with 39-40 million reads from SNU-001, Yuwolcho, PI201234, and YCM334 (Table 1). After quality trimming with Sickle version 1.0, the collected data were reduced to 3.6-3.7 Gb with 35-37 million reads of 100-102 bp read length for Jeju, Tean, and LAM32; 5.3 Gb with 67 million reads averaging 79.5 bp for CM334; and 3.4-3.5 Gb with 39-40 million reads averaging 87-88 bp for SNU-001, Yuwolcho, PI201234, and YCM334 (Table 1).

Alignment of Each Accession to the Reference Sequence

The total acquired reference sequence of 21,665 kb was de novo assembled from 31,196 contigs derived from Bukang ESTs, with an average contig length of 696 bp. There was a total of 1,071 kb of interspersed repeat elements (5,216 elements). Unclassified repeats accounted for 626 kb of this. Of the remaining repeat sequence, the largest portion (278 kb) was annotated as long terminal repeats (LTR), which are composed of several hundred base pairs. Long interspersed nuclear elements (LINEs), which encode a reverse transcriptase (RT) and other proteins, accounted for 118 kb. In addition, there was 49 kb of DNA transposable elements, which tend to have short inverted repeats at each end. In addition to these interspersed repeat elements, 102 kb and 103 kb were marked as simple repeat and low complexity regions, respectively. Therefore, a total of 1,276 kb (5.9%) was masked as repeat sequences.

The trimmed reads of eight accessions (Table 1) were aligned to the reference sequence and the range of alignment ratio between each accession and the reference was 52.6-70.5% (Table 2).

Table 2. Results of alignment of each accession to the reference sequence.

Cultivar	Trimmed reads	Aligned reads	Alignment ratio (%)
Jeju	36,990,461	19,465,349	52.62
LAM32	35,239,208	20,122,901	57.1
Tean	37,165,176	20,983,023	56.46
CM334	66,591,676	36,054,433	54.14
SNU-001	39,338,990	24,801,440	63.05
Yuwolcho	39,381,505	26,073,255	66.21
PI201234	39,637,637	27,929,380	70.46
YCM334	39,081,800	25,936,924	66.37

SNP Discovery and SNP Filtering

Sequences with read depth over 25x were used for SNP discovery, and those that were obviously distinguishable and different among accessions were defined as SNPs. Based on these criteria, a total of 58,151 SNPs were identified. From these, SNPs with only two types of genotypes and two alleles were filtered out. Among the remaining 57,502 SNPs, 33,315 were recognized as distinguishable in at least six different accessions (Table 3). In the next step, SNPs that showed a uniform segregation ratio were chosen. Among the 33,315 SNPs tested, 4,508 segregated 4:4 or 3:5 in the eight accessions. Finally, from those 4,508 SNPs, 1,910 were selected based on not having any other SNPs within 60 bp in either direction (Table 3).

Development and Validation of SNP Markers

Among 1,910 SNPs, a total of 1,282 were positioned on the hot pepper map produced by UC Davis. From these, we further selected 412 SNPs that showed clear and repeatable polymorphism, and that were evenly distributed over a ~3-cM interval in each chromosome (Supplementary Table 1). The linkage map showing the location of the 412 SNPs is presented in Fig. 1 and the SNP number per chromosome is given in Table 4. The entire length of the genetic map was 1,460.6 cM. There was an average of 34 SNPs in each linkage group, with the P1_Wild (177.1 cM) linkage group including the most SNPs. The P3 linkage group had the highest density (0.40 SNP/cM). The fewest

Table 3. SNP filtering process.

Filtering criteria	No. of SNPs remaining	No. of filtered SNPs	Filtering percentage (%)
Depth filtering (> 25 X)	58,151		
Genotype/allele	57,502	649	1.11
No. of distinguishable accessions	33,315	24,187	42.06
Segregation ratio	4,508	28,807	86.46
Adjacent SNP	1,910	2,598	57.63

Filtering percentage = (No. of SNPs removed by filterNo. of SNPs tested) × 100.

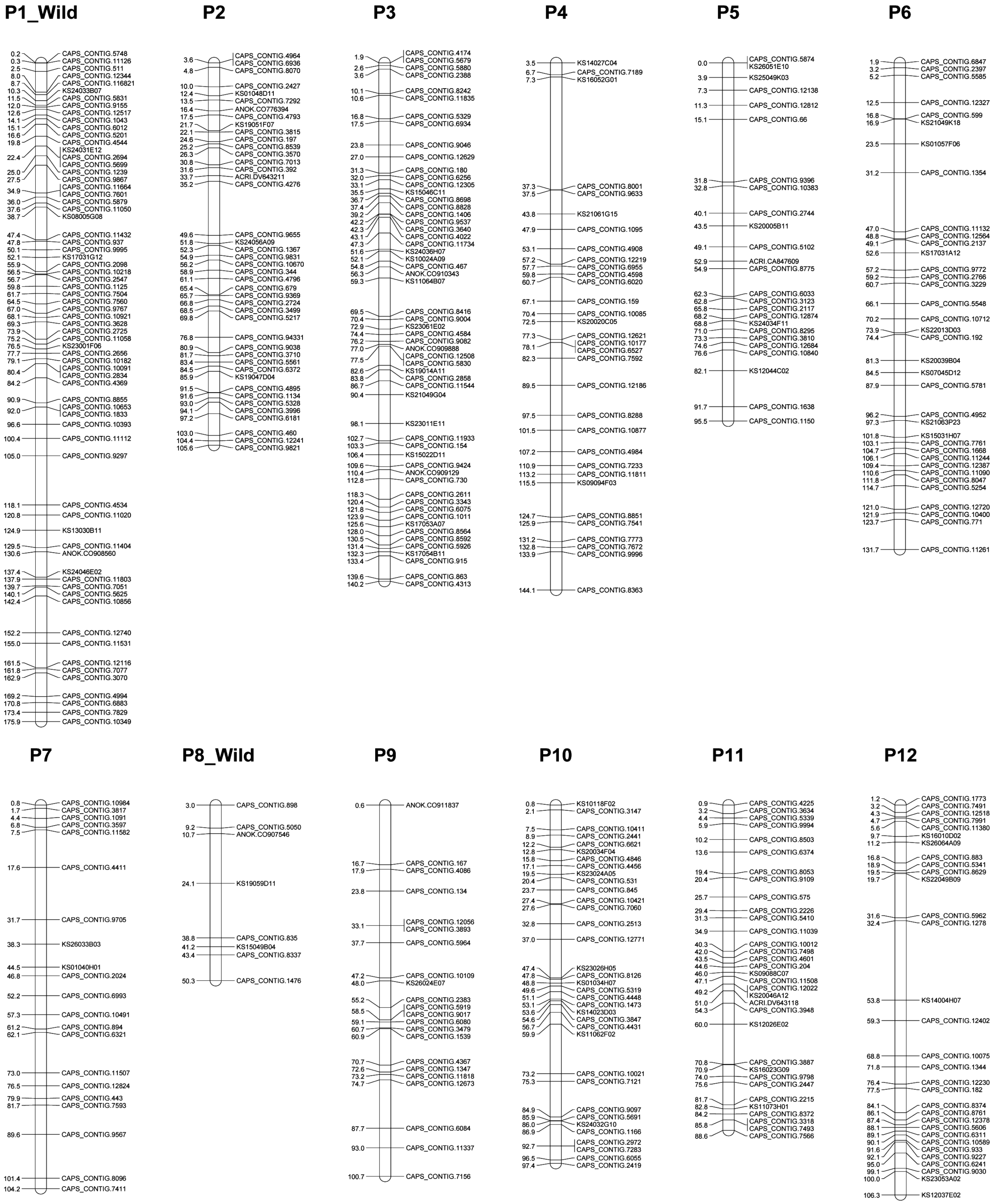


Fig. 1. Location of 412 SNP markers on pepper linkage map.

Table 4. The number of SNPs and density in each pepper linkage group.

Chromosome	SNPs	Size (cM)	SNP density (SNP/cM)
P1_Wild	69	177.1	0.39
P2	43	113.8	0.38
P3	57	141.6	0.40
P4	32	144.1	0.22
P5	25	101.6	0.25
P6	36	136.6	0.26
P7	21	104.2	0.20
P8_Wild	8	50.3	0.16
P9	22	110.2	0.20
P10	35	98.0	0.36
P11	33	90.0	0.37
P12	31	106.3	0.29
Total	412	1460.6	0.28

SNPs mapped to linkage group P8_Wild. The number of SNPs per cM ranged from 0.16 to 0.40, with an average of 0.28 SNPs per cM. Accordingly, there an average of 1 SNP per 3.55-cM interval in the genetic map (Table 4 and Fig. 1).

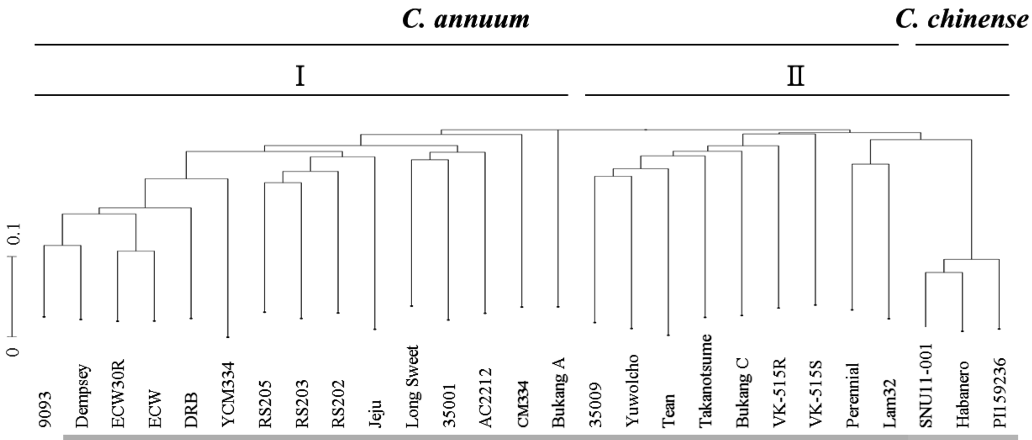
To validate the 412 SNP markers, 24 *C. annuum* and 3 *C. chinense* accessions (Supplementary Table 2) were tested for diversity of pepper genotypes (Fig. 2). Between *C. annuum* accessions, the average number of SNPs was 143, ranging from 23 (ECW vs. ECW30R) to 205 (Takanotsume vs. YCM334). By contrast, between bell-type accessions of *C. annuum* (9003, Dempsey, ECW30R, ECW), the average number of SNPs was 51, ranging from 23 (ECW vs. ECW30R) to 69 (DRB vs. ECW). *C. chinense* accessions showed an average of 23 SNPs, ranging from 17 (Habanero vs. SNU11-001) to 27 (Habanero vs. PI159236). The SNP number between *C. annuum* and *C. chinense* accessions averaged 179, ranging from 149 (VK-515S vs Habanero) to 209 (Dempsey vs PI159236). Overall, the average SNP number between all *Capsicum* accessions was 150, ranging from 17 (Habanero vs SNU11-001) to 209 (Dempsey vs PI159236). Based on the genetic similarity results, a cluster dendrogram placed the 27 pepper accessions into two main clusters and clearly differentiated each accession (Fig. 2). The first main cluster (I) comprised 15 different *C. annuum* accessions (9093, Dempsey, ECW30R, ECW, DRB, YCM334, RS205, RS203, RS202, Jeju, LongSweet, 35001, AC2212, CM334, Bukang A). The second cluster (II) consisted of 12 different accessions including seven *C. annuum* accessions (35009, Yuwolcho, Tean, Takanotsume, Bukang C, VK-515R, and VK515S), two wild species of *C. annuum* accessions (Perennial and Lam 32) and three *C. chinenese* accessions (PI159236, Habanero, and SNU11-001).

Discussion

Various approaches have been used to find SNPs in hot pepper, such as targeting sequences using COSII markers (Jung et al., 2010) or PCR using primers based on BAC sequences (Yang et al., 2009). Recently, ESTs have been widely used to find SNPs in crop varieties including tomato and barley because of their abundance and easy accessibility to the data (Kota et al., 2001; Labate and Baldo, 2005). The present work demonstrates that EST-derived SNP discovery using NGS is advantageous in hot pepper as well.

We prepared the reference sequence from Bukang ESTs using an approach similar to that described by Nicoli et al. (2012). However, we employed different SNP filtering methods to find valuable SNPs more effectively. By preparing a lengthy reference sequence after de novo assembly, we generated an alternative to WGS for reference sequence alignment to identify SNPs. Repeat areas are distributed throughout the genome in hot pepper. Therefore when preparing a reference sequence, repeat masking should be performed to find more accurate SNPs. In NGS for Jeju, LAM32, and Tean, parts of some reads contained low quality data and quality trimming resulted in small reductions in bases but not read number. However, for CM334, there was large amount of reduction due to low quality in both bases and reads. The sequences of SNU-001, Yuwolcho, PI201234, and YCM334 were high quality and did not show no much difference before and after trimming. Most accessions aligned to the reference with 50-70% of reads alignment. The variations might be due to the different genetic relationships between the accessions and the reference.

Overall, we identified many SNPs by aligning NGS sequence to the reference. However, the SNPs were not all equally valuable. Several factors can lead to false SNP findings, including base calling errors from NGS (Nielsen et al., 2011), miss-calls including overlapping genotypes (Anney et al., 2008), and false discovery of polymorphic SNPs that are actually monomorphic (Pettersson et al., 2008). To address this, we performed quality filtering of the SNPs. Our in-house SNP filtering process significantly decreased the SNP numbers: starting from 58,151 SNPs, the SNPs were filtered down to 1,910. After positioning the SNPs on the genetic linkage map, developing and testing SNP markers using the Fluidigm® EP1™ genotyping system, 412 SNP markers were finally



9093	Dempsey	ECW30R	ECW	DRB	YCM334	RS205	RS203	RS202	Jeju	Long Sweet	35001	AC2212	CM334	Bukang A	35009	Yuwolcho	Tean	Takanotsume	Bukang C	VK-515R	VK-515S	Perennial	Lam32	SNU11-001	Habanero	PI159236	
	25	50	51	54	106	91	106	121	133	127	133	143	138	175	165	193	195	184	178	163	137	157	186	192	184	192	9093
		58	58	54	108	94	108	120	130	127	133	145	140	176	162	189	203	186	179	165	145	156	180	196	192	209	Dempsey
			23	66	102	97	104	114	131	122	135	147	142	168	170	186	187	173	170	161	132	161	168	175	185	181	ECW30R
				69	104	99	109	110	139	133	135	156	139	178	173	180	189	191	176	168	138	172	190	188	187	178	ECW
					121	93	108	121	133	130	145	149	146	177	159	189	194	182	176	159	127	140	181	184	180	194	DRB
						115	135	143	166	157	160	168	142	192	192	202	195	205	195	188	135	173	187	198	200	189	YCM334
							42	55	88	91	103	100	132	102	128	144	137	147	132	98	97	134	132	150	151	153	RS205
								73	117	105	115	126	158	121	139	164	154	167	153	132	136	153	166	171	168	181	RS203
									112	111	126	114	146	95	141	160	146	158	145	123	122	156	157	173	167	173	RS202
										129	142	146	168	138	169	172	182	184	176	155	150	162	180	204	201	205	Jeju
											109	113	168	150	157	178	166	160	154	148	136	161	166	171	173	178	Long Sweet
												131	164	144	161	185	166	173	158	164	134	171	176	182	175	185	35001
													150	135	141	147	144	172	157	149	141	169	177	171	171	177	AC2212
														169	180	162	169	154	145	157	134	145	153	162	162	152	CM334
															135	151	156	151	145	150	133	163	159	189	181	187	Bukang A
																109	118	143	137	134	145	158	188	188	176	193	35009
																	121	125	128	141	147	163	183	204	202	190	Yuwolcho
																		135	153	137	140	181	197	205	203	199	Tean
																			132	138	120	138	134	171	177	175	Takanotsume
																				136	118	148	156	173	172	170	Bukang C
																					113	146	156	182	176	185	VK-515R
																						106	125	154	149	150	VK-515S
																							110	163	157	163	Perennial
																								168	172	167	Lam32
																									17	24	SNU11-001
																										27	Habanero
																											PI159236

▨ *C. annuum* vs *C. annuum*
▨ *C. annuum* vs *C. chinense*
▨ *C. chinense* vs *C. chinense*

Fig. 2. Polymorphism survey of the 412 SNP markers in 27 *Capsicum* cultivars and cluster dendrogram. SNP numbers are indicated for the comparisons between the *Capsicum* accessions indicated on the horizontal and vertical axes. Blue: SNP number for comparison between *C. annuum* accessions, red: SNP number for comparison between *C. annuum* and *C. chinense*, green: SNP number for comparison between *C. chinense* accessions.

selected and validated using 27 *Capsicum* accessions.

For MABC, background selection focuses on both reduction of donor segments around target genes and recurrent parent genome recovery. Successful MABC depends on genome size, population size, marker density in the map and the use of high throughput marker systems. Recently, Herzog and Frisch (2013) conducted computer simulations of MABC in genetic models of sugar beet, rye, sunflower and rapeseed, using the 10% quantile (Q10) value, the arithmetic mean and the standard deviation of the probability distribution of the proportion of recipient genome in the entire genome of selected individuals (as a percentage), for determining every backcross generation to measure recurrent parent genome recovery. Based on their data, the optimum designs, which minimize the required number of marker data points for target Q10 values of 96-99% in generation BC_2 or BC_3 in the model plants, employed marker densities of 2-20 cM intervals between markers and a population size of 50-100. In this study, the entire length of the genetic map covered by the SNP markers was 1,460.6 cM, suggesting that 73-730 markers (for 2-20 cM intervals) are needed for MABC in pepper. Our marker set produced an average of 150 SNPs between 27 accessions (corresponding to ~10 cM intervals), implying that our newly developed SNP markers should be useful for MABC between 27 accessions. SNP numbers between *C. annuum* and *C. chinense* were much higher than those between accessions from the same species such as bell types of *C. annuum* and *C. chinense*. This result implies that MABC could be more difficult in closely-related accessions than in distant accessionss. Despite this, our new SNP markers clearly distinguished 27 *Capsicum* accessions.

Among the accessions tested, AC2212 was positioned in the *C. annuum* cluster, rather than in the *C. chinense* cluster. AC2212 was originally classified as *C. chinense* based on agronomic information from the Centre for Genetic Resources, the Netherlands (http://applicaties.wageningenur.nl/applications/cgngenis/; Wahyuni et al., 2011). However, Wahyuni et al. (2013) reported out that AC2212 likely belongs to *C. annuum* instead of *C. chinense*, based on AFLP marker analysis. This result thus indicates that our newly developed SNP markers are accurate and can be used for the identification of diverse *Capsicum* accessions.

In conclusion, our SNP markers derived from transcriptome sequences will be valuable tools for MABC. In addition, the markers can be used for genetic mapping, comparative mapping and genetic diversity analyses in pepper and related species.

Literature Cited

Anney, R.J., E. Kenny, C.T. O'Dushlaine, and J. Lasky-Su. 2008. Non-random error in genotype calling procedures: implications for family-based and case-control genome-wide association studies. Am. J. Med. Genet. B. 147B:1379-1386.

Ashrafi, H., T. Hill, K. Stoffel, A. Kozik, J. Yao, S.R. Chin-Wo, and A. Van Deynze. 2012. De novo assembly of the pepper transcriptome (*Capsicum annuum*): A benchmark for in silico discovery of SNPs, SSRs and candidate genes. BMC Genomics 13:571.

Blum, E., K. Liu, M. Mazourek, E.Y. Yoo, M. Jahn, and I. Paran. 2002. Molecular mapping of the *C* locus for presence of pungency in *Capsicum*. Genome 45:702-705.

Flicek, P. and E. Birney. 2009. Sense from sequence reads: Methods for alignment and assembly. Nat. Methods 6:S6-S12.

Goff, S.A., D. Ricke, T.H. Lan, G. Presting, R. Wang, M. Dunn, J. Glazebrook, A. Sessions, P. Oeller, H. Varma, D. Hadley, D. Hutchison, C. Martin, F. Katagiri, B.M. Lange, T. Moughamer, Y. Xia, P. Budworth, J. Zhong, T. Miguel, U. Paszkowski, S. Zhang, M. Colbert, W.L. Sun, L. Chen, B. Cooper, S. Park, T.C. Wood, L. Mao, P. Quail, R. Wing, R. Dean, Y. Yu, A. Zharkikh, R. Shen, S. Sahasrabudhe, A. Thomas, R. Cannings, A. Gutin, D. Pruss, J. Reid, S. Tavtigian, J. Mitchell, G. Eldredge, T. Scholl, R.M. Miller, S. Bhatnagar, N. Adey, T. Rubano, N. Tusneem, R. Robinson, J. Feldhaus, T. Macalma, A. Oliphant, and S. Briggs. 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science 296:92-100.

Herzog, E. and M. Frisch. 2013. Efficient marker-assisted backcross conversion of seed-parent lines to cytoplasmic male sterility. Plant Breed. 132:33-41.

Imelfort, M., C. Duran, J. Batley, and D. Edwards. 2009. Discovering genetic polymorphisms in next-generation sequencing data. Plant Biotechnol. J. 7:312-317.

Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, and T.L. Madden. 2008. NCBIBLAST: A better web interface. Nucleic Acid Res. 36:W5-W9.

Jones, E., W.-C. Chu, M. Ayele, J. Ho, Ed Bruggeman, K. Yourstone, A. Rafalski, O.S. Smith, M.D. McMullen, C. Bezawada, J. Warren, J. Babayev, S. Basu, and S. Smith. 2009. Development of single nucleotide polymorphism (SNP) markers for use in commercial maize (*Zea mays* L.) germplasm. Mol. Breed. 24:165-176.

Jung, J.K., S.W. Park, W.Y. Liu, and B.C. Kang. 2010. Discovery of single nucleotide polymorphism in *Capsicum* and SNP markers for cultivar identification. Euphytica 175:91-107.

Kang, B.C., S.H. Nahm, J.H. Huh, H.S. Yoo, J.W. Yu, M.H. Lee, and B.D. Kim. 2001. An interspecific (*Capsicum annuum* x *C. chinese*) F_2 linkage map in pepper using RFLP and AFLP markers. Theor. Appl. Genet. 102:531-539.

Kim, H.J., K.H. Baek, S.W. Lee, J. Kim, B.W. Lee, H.S. Cho, W.T. Kim, D. Choi, and C.G. Hur. 2008. Pepper EST database: comprehensive in silico tool for analyzing the chili pepper (*Capsicum annuum*) transcriptome. BMC Plant Biol. 8:101.

Kota, R., R.K. Varshney, T. Thiel, K.J. Dehmer, and A. Graner. 2001. Generation and comparison of EST-derived SSRs and SNPs in barley (*Hordeurn vulgare* L.). Hereditas 135:145-151.

Labate, J.A. and A.M. Baldo. 2005. Tomato SNP discovery by EST mining and resequencing. Mol. Breed. 16:343-349.

Lee, M.J., B.M. Popkin, and S. Kim. 2002. The unique aspects of the nutrition transition in South Korea: The retention of healthful elements in their traditional diet. Public Health Nutr. 5:197-203.

Li, H. and R. Durbin. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754-1760.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, and 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078-2079.

McPherson, J.D. 2009. Next-generation gap. Nat. Methods 6:S2-S5.

Metzker, M.L. 2005. Emerging technologies in DNA sequencing. Genome Res. 15:1767-1776.

Metzker, M.L. 2010. Sequencing technologies - The next generation. Genetics 11:31-46.

Nicolai, M., C. Pisani, J.P. Bouchet, M. Vuylsteke, and A. Palloix. 2012. Discovery of a large set of SNP and SSR genetic markers by high-throughput sequencing of pepper (*Capsicum annuum*). Genet. Mol. Res. 11:2295-2300.

Nielsen, R., J.S. Paul, A. Albrechtsen, and Y.S. Song. 2011. Genotype and SNP calling from next-generation sequencing data. Nat. Rev. Genet. 12:443-451.

Paran, I., J.R. van der Voort, V. Lefebvre, M. Jahn, L. Landry, M. van Schriek, B. Tanyolac, C. Caranta, A.B. Chaim, K. Livingstone, A. Palloix, and J. Peleman. 2004. An integrated genetic linkage map of pepper (*Capsicum* spp.). Mol. Breed. 13:251-261.

Perrier, X., A. Flori, and F. Bonnot. 2003. Data analysis methods, p. 43-46. In: P. Hamon, M. Seguin, X. Perrier, and J.C. Glaszmann. (eds.). Genetic diversity of cultivated tropical plants. Gifield Science Publishers, Montpellier.

Pettersson, F., A.P. Morris, M.R. Barnes, and L.R. Cardon. 2008. Goldsurfer2 (Gs2): A comprehensive tool for the analysis and visualization of genome wide association studies. BMC Bioinformatics 9:138.

Pompanon, F., A. Bonin, E. Bellemain, and P. Taberlet. 2005. Genotyping errors: Causes. Consequences and solutions. Nat. Rev. Genet. 6:847-859.

Rudd, S. 2003. Expressed sequence tags: alternative or complement to whole genome sequences? Trends Plant Sci. 8:321-329.

Shao, G.C., Z.Y. Zhang, N. Liu, S.E. Yu, and W.G. Xing. 2008. Comparative effects of deficit irrigation (DI) and partial rootzone drying (PRD) on soil water distribution, water use, growth and yield in greenhouse grown hot pepper. Sci. Hortic. 119:11-16.

Shendure, J. and H.L. Ji. 2008. Next-generation DNA sequencing. Nat. Biotechnol. 26:1135-1145.

Teo, Y.Y., A.E. Fry, T.G. Clark, E.S. Tai, and M. Seielstad. 2007. Data sheet: Sentrix HumanHap550 genotyping BeadChip. Ann. Hum. Genet. 71:701-703.

The Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796-815.

Varshney, R.K., S.N. Nayak, G.D. May, and S.A. Jackson. 2009. Next-generation sequencing technologies and their implications for crop genetics and breeding. Trends Biotechnol. 27:522-530.

Vignal, A., D. Milan, M. SanCristobal, and A. Eggen. 2002. A review on SNP and other types of molecular markers and their use in animal genetics. Genet. Sel. Evol. 34:275-305.

Wahyuni, Y., A.R. Ballester, E. Sudarmonowati, R.J. Bino, and A.G. Bovy. 2011. Metabolite biodiversity in pepper (*Capsicum*) fruits of thirty-two diverse cultivars: Variation in health-related compounds and implications for breeding. Phytochemistry 72: 1358-1370.

Wahyuni, Y., A.R. Ballester, Y. Tikunov, R.C.H. de Vos, K.T.B. Pelgrom, A. Maharijaya, E. Sudarmonowati, R.J. Bino, and A.G. Bovy. 2013. Metabolomics and molecular marker analysis to explore pepper (*Capsicum* sp.) biodiversity. Metabolomics 9:130-144.

Weiss, E.A. 2002. Spice crops. CABI Publishing International, Oxon, UK p. 7-22.

Wittenberger, T., H.C. Schaller, and S. Hellebrand. 2001. An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. J. Mol. Biol. 307:799-813.

Yang, H.B., W.Y. Liu, W.H. Kang, M. Jahn, and B.C. Kang. 2009. Development of SNP markers linked to the *L* locus in *Capsicum* spp. by a comparative genetic analysis. Mol. Breed. 24:433-446.

Yang, H.B., W.Y. Liu, W.H. Kang, J.H. Kim, H.J. Cho, J.H. Yoo, and B.C. Kang. 2012. Development and validation of *L* allele-specific markers in *Capsicum*. Mol. Breed.30:819-829.

Yi, G.B., J.M. Lee, S. Lee, D. Choi, and B.D. Kim. 2006. Exploitation of pepper EST-SSRs and an SSR-based linkage map. Theor. Appl. Genet. 114:113-130.

Yoo, E.Y., S. Kim, Y.H. Kim, C.J. Lee, and B.D. Kim. 2003. Construction of a deep coverage BAC library from *Capsicum annuum*, ‘CM334’. Theor. Appl. Genet. 107:540-543.