Rapid Construction of a Linkage Map using High-efficiency Genome Scanning/AFLP and RAPD, Based on an Intraspecific, Doubled-haploid Population of *Capsicum annuum*

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A genetic linkage map of the sweet pepper (*Capsicum annuum* L.) using an intraspecific doubled-haploid (DH) population was primarily constructed by amplified fragment-length polymorphism (AFLP) using the high efficiency genome scanning (HEGS) system and random amplified polymorphic DNA (RAPD). Linkage analysis was done using a total of 518 molecular markers that consisted of 382 AFLP, 122 RAPD, 3 RFLP, 7 SCAR and 4 CAPS markers. The linkage groups consisted of 11 large linkage groups (56.7 to 118.5 cM) and 5 small linkage groups (1.8 to 33.1 cM), covering a total distance of 1043.1 cM with an average distance between 224 framework markers of 4.6 cM. AFLP markers could be developed quickly using HEGS, even in an intraspecific DH population in which it is generally difficult to detect polymorphisms in comparison with interspecific crossing populations. The map was constructed essentially in two months. Linkage analysis also provided three AFLP markers and an RAPD marker linked to PMMoV resistance (L^3), and an AFLP marker linked to C that was required for expression of pungency. A closer marker linked to C , Plastid-lipid-Associated Protein-simple sequence repeat (PAP-SSR), a microsatellite marker linked to C , was found at a distance of 0.6 cM. We examined the usefulness of PAP-SSR with three species in *Capsicum* using fragment analysis and nucleotide sequences, many alleles were found at this locus. The results suggested that these markers could be effective in marker-assisted selection (MAS) programs for sweet pepper breeding purposes.

Key Words: *Capsicum*, sweet pepper, PMMoV, pungency, high efficiency genome scanning (HEGS), amplified fragment length polymorphism (AFLP), linkage map, doubled-haploid (DH) population.

Introduction

The genus *Capsicum* belongs to Solanaceae. This genus contains five species, i.e., *C. annuum*, *C. frutescens*, *C. baccatum*, *C. pubescens* and *C. chinense*. *C. annuum* is known as pepper, sweet pepper or paprika (*C. annuum* L. var. *angulosum* Miller or var. *grossum* Bailey), and is cultivated globally for spices, vegetables, colorants and medical supplies. In recent years, DNA markers linked to various agriculturally important characters have been developed in *Capsicum* species. Using these linkage markers marker-assisted selection (MAS) has now become possible in some traits. However, to widen the possibility of MAS in *Capsicum* breeding, a method for rapid construction of linkage

maps should be developed.

Restriction fragment-length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990), simple sequence repeat (SSR also designated as microsatellites) (Tautz 1989, Weber and May 1989) and amplified fragment-length polymorphism (AFLP) (Vos *et al.* 1995) are generally used to construct the linkage map. However, detection of RFLP requires a great deal of labor and much more DNA than the other PCR-based methods (e.g. RAPD, SSR and AFLP).

On the contrary, RAPD is simpler than other techniques, its reproducibility is reportedly poor. Although AFLP can detect many bands at once and can be mapped efficiently, labeled primers with radioactive isotope or fluorescent chemicals are needed to detect it. However, the procedure in this technique is complicated. Recently Kawasaki and Murakami (2000) developed a high-efficiency genome scanning (HEGS) system, a modified detection system for

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AFLP. The system uses small electrophoresis units with non-labeled primers, and can also be used for detection of SSR polymorphism. Combining HEGS and RAPD, genetic maps can be easily constructed with high cost performance.

In *Capsicum*, Tanksley *et al.* (1988), Prince *et al.* (1993), Lefebvre *et al.* (1995) and Livingstone *et al.* (1999) have constructed genetic maps using RFLP with tomato probes. Kang *et al.* (2001) constructed a genetic map using 150 RFLP markers and 430 AFLP markers on an interspecific F_2 population. Furthermore, DNA markers linked to various useful characteristics have been identified. These markers include disease resistance to pepper mild mottle virus (PMMoV; L^4) (Matsunaga *et al.* 2003), cucumber mosaic virus (Ben *et al.* 2001a), tomato spotted wilt virus (Moury *et al.* 2000) and potato virus Y (Caranta *et al.* 1999), fruit shape (Ben *et al.* 2001b), pungency (Blum *et al.* 2002) and mature fruit color (Popovsky and Paran 2000, Huh *et al.* 2001). Moreover, we have developed SCAR markers linked to PMMoV resistance (L^3) (Sugita *et al.* 2004).

We then tried to construct the linkage map using AFLP/HEGS, RAPD for comparison with AFLP/HEGS and RFLP based on an intraspecific DH population derived from a crossing between 'K9-11' and 'AC2258'. Furthermore, we mapped the L^3 and the C locus required for expression of pungency and verified the possibility of securing many DNA markers linked to each loci.

Materials and Methods

Plant materials and DNA extraction

A DH population ($n=176$) was obtained by anther culture of an F_1 hybrid between 'K9-11' (*C. annuum* L.) and 'AC2258' (*C. annuum* L.). 'K9-11' harbors the L^3 gene derived from PI159236, and is a bell-like sweet pepper (Yanokuchi *et al.* 1993, Sugita *et al.* 2004). It is susceptible to phytophthora blight and has no pungency, and its immature fruit color is dark green. 'AC2258' is resistant to phytophthora blight and is pungent. The color of the immature fruit of this line is light yellow. Anther culture was performed using the method described by Dumas de Vaulx *et al.* (1981) with a minor modification (Todoroki *et al.* 1994, Yamaguchi *et al.* 1994).

Genomic DNA was extracted from young leaf tissues using the CTAB method as described by Murray and Thompson (1980), and Nucleon PHYTOPURE (Amersham LIFE SCIENCE, Buckinghamshire).

RAPD analysis

DNA samples were screened with a total of 2,397 combinations of two arbitrary 10 and 12 mer primers (Operon Technologies, Calif; BEX, Tokyo). The amplification conditions were adopted for RAPD analysis with minor modifications. The PCR reaction mixture consisted of 20 ng of template DNA, $1 \times$ reaction buffer (10 mM Tris HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl) (Takara Biomedicals, Otsu), 200 μ M of dNTPs, 0.2 μ M of random primers and two units of

rTaq DNA polymerase (Takara Biomedicals, Otsu) in a total reaction volume of 20 μ l. DNA amplification was performed in a Perkin-Elmer GeneAmp PCR System 9700 and 9600. The mixture was initially denatured at 95°C for 2 min, followed by 45 cycles of PCR amplification with denaturation at 94°C for 1 min, primer annealing at 35°C for 2 min, primer extension at 72°C for 3 min, and finally incubated at 72°C for 7 min. The PCR products were separated by electrophoresis on a 2% agarose gel (SeaKem GTG agarose, FMC BioProducts, Rockland) with $0.5 \times$ TBE buffer (44.5 mM Tris-borate, 1 mM EDTA), and stained with ethidium bromide.

AFLP analysis

The amplification conditions were adopted for AFLP analysis and initially described by Vos *et al.* (1995). The electrophoresis conditions and the band-detections were adopted for HEGS as described by Kawasaki and Murakami (2000) and Kawasaki *et al.* (2003). Genomic DNA of 250 ng digested with *MseI* 5 unit/*EcoRI* 5 unit, *MseI* 5 unit/*PstI* 5 unit or *MseI* 5 unit/*XbaI* 5 unit. Digested fragments were ligated to each adaptor of restriction enzyme combination. The first amplification was performed using pre-amplification primers (Table 1). The first PCR products diluted with 1/10 TE (1 mM Tris hydroxymethyl aminomethane, 0.1 mM disodium dihydrogen ethylenediamine tetraacetate dehydrate, pH 8.0) buffer to 1/100 were used as template DNA for second amplification. The second amplification was performed using selective primers (Table 1) in a 10 μ l total reaction

Table 1. Nucleotide sequences of the primers and adaptors used in AFLP analysis

Primers/Adaptors	Nucleotide sequences
<i>MseI</i> adapter 1	5'-gac gat gag tcc tga g-3'
<i>MseI</i> adapter 2	5'-tac tca gga ctc at-3'
<i>EcoRI</i> adapter 1	5'-ctc gta gac tgc gta cc-3'
<i>EcoRI</i> adapter 2	5'-aat tgg tac gca gtc tac-3'
<i>PstI</i> adapter 1	5'-ctc gta gac tgc gta cat gca-3'
<i>PstI</i> adapter 2	5'-tgt acg cag tct ac-3'
<i>XbaI</i> adapter 1	5'-ctc gta gac tgc gta cc-3'
<i>XbaI</i> adapter 2	5'-cta ggg tac gca gtc tac-3'
<i>MseI</i> primer (+1)	5'-gat gtg tcc tga gta ac-3'
<i>EcoRI</i> primer (+1)	5'-gac tgc gta cca att ca-3'
<i>PstI</i> primer (+1)	5'-gac tgc gta cat gca gg-3'
<i>XbaI</i> primer (+1)	5'-gac tgc gta ccc tag ac-3'
<i>MseI</i> primer (+3)	5'-gat gtg tcc tga gta ac + aa(M1), ac(M2), ag(M3), at(M4), ca(M5), cc(M6), cg(M7), ct(M8), ga(M9), gc(M10), gg(M11), gt(M12), ta(M13), tc(M14), tg(M15), tt(M16)-3'
<i>EcoRI</i> primer (+3)	5'-gac tgc gta cca att ca + aa(E1), ac(E2), ag(E3), at(E4), ca(E5), cc(E6), cg(E7), ct(E8), ga(E9), gc(E10), gg(E11), gt(E12), ta(E13), tc(E14), tg(E15), tt(E16)-3'
<i>PstI</i> primer (+3)	5'-gac tgc gta cat gca gg + ga, gt, ta, tc, tg, tt-3'
<i>XbaI</i> primer (+3)	5'-gac tgc gta ccc tag ac + cc(X6), cg(X7), ct(X8), ga(X9), gc(X10), gg(X11)-3'

Table 2. Nucleotide sequences and information of the primers used in this study

Markers ¹⁾	Primers	Description	Forward primer	Reverse primer	Reference
CAPS	CCS	<i>C. annuum</i> capsanthin/capsorubin synthase gene	5'-gggtacatgggtgctcctag-3'	5'-gagggacaagagtgaggcag-3'	X77289
CAPS	Sn-2	<i>C. annuum</i> <i>Sn-2</i> gene	5'-tgaagtgtggaggagcctg-3'	5'-gcgtcgattgatgggcca-3'	X79231
CAPS	LCYB	<i>C. annuum</i> lycopene Beta-cyclase	5'-gcacctgttgggaaaatggatagc-3'	5'-gatccagataagtcgaattcattc-3'	X86221
CAPS	N4F6R6	Nicotiana glutinosa virus resistance (N) gene	5'-aggctctgggataagggaactacca-4'	5'-tgatgggtggaaccaactcgggac-3'	U15605
SSR	PAP-SSR	<i>C. annuum</i> fibrillin gene; <i>C. annuum</i> PAP gene	5'-ctccaaactacccctggattttc-3'	5'-gtcaatcccaatagacttttaaatgtgc-3'	X77290, AJ131456
SCAR	ASC002	CAPS marker linked to <i>Pvr4</i> in pepper ²⁾	5'-cgaagagagaagggtc-3'	5'-tcagggttaggttatt-3'	Caranta <i>et al.</i> 1999
SCAR	H6-4	SCAR marker linked to <i>Bs3</i> in pepper	5'-aagcttaacacatctttc-3'	5'-ttaaaccgttgctgttac-3'	Pierre <i>et al.</i> 2000
SCAR	H17-52	SCAR marker linked to <i>Bs3</i> in pepper	5'-cttatataataacagaggcg-3'	5'-gtaatgagccacactaac-3'	Pierre <i>et al.</i> 2000
SCAR	S19	CAPS marker linked to <i>Bs2</i> in pepper ²⁾	5'-ctgaccagccaacatgg-3'	5'-ctgaccagccctttcaactac-3'	Tai <i>et al.</i> 1999
SCAR	PMFR11	SCAR marker linked to <i>L3</i> in pepper	5'-ctgcagaacaacaatgcacg-3'	5'-ggactgcagaggagggaagc-3'	Sugita <i>et al.</i> 2004
SCAR	PMFR21	SCAR marker linked to <i>L3</i> in pepper	5'-gccaaatgtgaattgaaac-3'	5'-ggactgcagaggagggaagc-3'	Sugita <i>et al.</i> 2004

¹⁾ CAPS markers and SSR marker were used to design gene sequence with previously established database. SCAR markers were from published literature.

²⁾ These CAPS markers were available as dominant SCAR markers on our DH population.

volume. The second PCR products were separated on 13% polyacrylamide gels under conditions of 250 V for 30 min and 420 V for 3 hrs in a small vertical electrophoresis unit of 26 × 26 cm (Nippon-eido, Tokyo). The gels were stained with SYBR Green I (BMA, Rockland) for 30 min, and were detected with a fluorescent image analyzer (FLA-3000G; Fuji Film, Tokyo).

RFLP, SCAR and CAPS analysis

Genomic DNA for RFLP analysis extracted by the CTAB method was digested with a restriction enzyme, XbaI, and separated by 0.8% agarose gel electrophoresis. The gels were transferred onto positively charged nylon membranes (Hybond N+, Amersham-Biosciences, Piscataway) by the capillary transfer method, and baked at 80°C for 2 hrs. Southern blot hybridization was performed with Gene Images Random-Prime Labelling and Detection System (Amersham-Biosciences, Piscataway) using the tomato probe provided by Cornell University. The SCAR markers were from previously published literature or databases (Table 2). The CAPS markers used were designed based on the known gene sequences with previously established databases (Table 2).

PMMoV inoculation and evaluation of pungency

An isolate of PMMoV (P1.2 pathotype), was collected in Miyazaki Prefecture and used in this study. Young leaves were excised and mechanically inoculated with the purified virus (20 ng/ml) by rubbing with #500 carborundum powder. The inoculated leaves were kept in a growth chamber under moist conditions at 25°C for 24 hrs with lighting. Local lesions grew on the leaves harboring the *L3* gene after three to six days. The pungency was evaluated by the organoleptic method, in which placenta tissue of the fruits was tasted 30 to 40 days after bloom.

Design of SSR primers and fragment analysis

The primers were designed based on the fibrillin gene (Accession No. X77290) that has been renamed the Plastid-lipid-Associated Protein (PAP) gene (Accession No. AJ131456 (Pozueta-Romero *et al.* 1997)) described by Blum

et al. (2002). The obtained PCR products were directly sequenced with an ABI310 sequencer (Applied Biosystems, Foster City). The primers labeled with fluorescent chemicals were newly designed from the obtained sequencing data, and the fragment length was analyzed with an ABI310 sequencer and GeneScan™ (Applied Biosystems, Foster City). The validity of the developed marker was examined with 28 accessions of three species in *Capsicum*. Furthermore, nucleotide sequences of PAP-SSR fragments on 23 accessions of three species in *Capsicum* were analyzed with an ABI310 sequencer by direct sequencing.

Data analysis

Linkage analysis using the DNA markers and two phenotypic loci was performed with the program MAPMAKER Ver. 3.0 (Lander *et al.* 1987) under the conditions of a maximum recombination fraction of 25 cM and a minimum LOD score above 3.0.

Fig. 1. AFLP analysis of an intraspecific DH population using primer combination M10/E12. M: Molecular marker (100 bp ladder). K: 'K9-11'. A: 'AC2258'. DH1-46: DH individuals. Arrowheads on the right side show polymorphic bands.

Results and Discussion

Construction of the linkage map

Although there was little difference in the polymorphic rate between the RAPD and AFLP analyses as for pepper, the average number of polymorphisms per primer using *EcoRI/MseI*, *PstI/MseI* and *XbaI/MseI* based on AFLP was respectively, 13.6, 8.2 and 12.7 times that based on RAPD. Polymorphisms can be detected more efficiently and quickly than by RAPD (Fig. 1). The average number of polymorphic bands and the average number of bands per primer were greatest for the combination of *EcoRI/MseI* on AFLP analysis (*EcoRI/MseI* > *XbaI/MseI* > *PstI/MseI*), and the polymorphic rate was high for *XbaI/MseI*. Having many polymorphisms per primer combination facilitates construction of the map in other crossing populations. When the combination of *EcoRI/MseI* was used, the bands were more crowded than in the case of combinations of *XbaI/MseI* and *PstI/MseI*, and difficult to be identified. Kang *et al.* (2001) have also reported that the combination of *EcoRI/MseI* generated

many AFLP markers than combinations of *PstI/MseI*, and the large number of polymorphic bands were difficult to score owing to the dense and sometimes overlapping banding patterns.

Linkage analysis was done using a total of 518 molecular markers that consisted of 382 AFLP, 122 RAPD, 3 RFLP, 7 SCAR and 4 CAPS markers. Since many markers were located at the same loci, 224 clear and good quality markers were used as the framework markers. The total of 16 linkage groups (LGs) consisting of 11 large LGs (56.7 to 118.5 cM) and five small LGs (1.8 to 33.1 cM), covering a total distance of 1043.1 cM with an average distance between 224 framework markers of 4.6 cM was obtained (Fig. 2). In addition, 42 out of 382 (11%) AFLP markers on this map were codominant. However, many AFLP and RAPD markers were located in the limited regions of LG 1 to LG10, LG12 and LG16, and these regions were presumed to be close to the centromere. Markers were gathered in limited regions on other maps of *Capsicum* (Livingston *et al.* 1999, Kang *et al.* 2001). On the contrary, this phenomenon

Fig. 2. A linkage map of the pepper on an intraspecific DH population, derived from F_1 crossed between 'K9-11' (*Capsicum annuum* L.) and 'AC2258' (*C. annuum* L.), using a total of 518 molecular markers consisting of 382 AFLP markers, 122 RAPD markers, three RFLP markers, seven SCAR markers and four CAPS markers. The map is constructed with 227 loci distributed into 16 linkage groups with a total genetic distance of 1040.5 cM. CA, CB, RA-Q and AA (CA $\times$ CA) are RAPD markers on the right side of each linkage group. TG designates the tomato RFLP markers. AFLP markers are named according to the combinations of selective primers in Table 1 used, followed by the numbers given in descending order of fragment length. *C* is a phenotypic loci for pungency, and *L*³ is a phenotypic loci for resistance to P_{1,2} in PMMoV. Map distances are in centiMorgans (cM) calculated by the Kosambi function, indicated on the left side of each linkage group.

Fig. 2. (continued)

was not so marked in other crops (*Hordeum vulgare* (Hori *et al.* 2003) and *Oryza sativa* (Kawasaki *et al.* 2003). It is considered to be the genome characteristic of this genus since it is recognized on other linkage maps of *Capsicum* (Livingston *et al.* 1999, Kang *et al.* 2001). In addition, there were 52 markers for which the distortion of the segregation ratio exceeded $P < 0.01$, and these regions were located mainly on LG3, LG9 and LG11. AFLP markers could be developed quickly and easily using HEGS, even in an intraspecific DH population in which it is generally difficult to detect polymorphisms in comparison with interspecific crossing populations. The map was constructed essentially in two months.

Moreover, by linkage analysis using the constructed linkage map and the result of PMMoV inoculation on a DH population, we could map the L^3 locus on the end of LG6 (Fig. 2). Since L^3 is reported to be located on the end of the 11th chromosome (Livingstone *et al.* 1999, Lefebvre *et al.* 2002), it is considered that LG6 on our map corresponds to the 11th chromosome. By linkage analysis using the constructed linkage map, we could map the C locus for pungency trait at the end of LG3 (Fig. 2). Since C is located on the middle of the second chromosome (Ben *et al.* 2001b), LG3 on our map is considered to correspond to the second chromosome.

Development of the DNA markers linked to L^3

An AFLP marker M10E6-4 (250 bp) cosegregated with the L^3 locus, an AFLP marker M1X9-2 (320 bp) and a RAPD marker AA6096 (680 bp) located on 1.1 cM from L^3 , and an AFLP marker M11E5-2 (1.2 kbp) at the opposite side of 2.9 cM across L^3 , were found (Fig. 2 and Table 3). Boukema (1982) reported that four pathotypes, P_0 , P_1 , $P_{1.2}$ and $P_{1.2.3}$, could be identified in PMMoV, while four types of allelic resistance genes, L^1 , L^2 , L^3 and L^4 , were found in *Capsicum* species. It was also reported that a clear gene-for-gene response was observed. As a result, the L^3 gene derived from PI159236 (*C. chinense*) is assumed to confer resistance to P_0 , P_1 and $P_{1.2}$, and the L^4 gene derived from PI260429 (*C. chacoense*) is assumed to confer resistance to P_0 , P_1 , $P_{1.2}$ and $P_{1.2.3}$, while many other accessions carrying only L^1 or L^2 genes are not resistant to $P_{1.2}$ and $P_{1.2.3}$. Fig. 3 shows the position of the L resistance to PMMoV and linked markers in *Capsicum*. An RFLP marker TG36 using a tomato probe, was mapped at 4.0 cM from L^3 with an interspecific DH population by Lefebvre *et al.* (1995). The SCAR markers, PMFR11₂₆₉, PMFR11₂₈₃ and PMFR21₂₀₀ were mapped at 4.0 cM from L^3 with an intraspecific DH population by Sugita *et al.* (2004) using a bulked segregant analysis method. A SCAR marker, WA31-1500S, was mapped at 1.5 cM from L^4 with an intraspecific F_2 population by Matsunaga *et al.* (2003). It is an L^4 -specific dominant marker, and is detected only in the accessions harboring the L^4 gene. In this study, three AFLP markers and an RAPD marker linked to L^3 were obtained at the locus closer than what is already reported. These closely linked molecular markers on both sides of the resistance gene, L^3 , enable us to select desirable geno-

Group 6 (Chr. 11)

Fig. 3. Position of the L locus for resistance to PMMoV and linked markers in *Capsicum*. Underlined markers, TG36 using a tomato probe, was mapped by Lefebvre *et al.* (1995). WA31-1500S, a SCAR marker was mapped by Matsunaga *et al.* (2003). PMFR11₂₆₉, PMFR11₂₈₃ and PMFR21₂₀₀, the SCAR markers were mapped by Sugita *et al.* (2004). Map distances are in centiMorgans (cM) calculated by the Kosambi function, indicated on the left side of the linkage map.

Table 3. Frequency distribution of PMMoV resistance dependent on markers in DH¹⁾ populations

DNA markers	resistant		susceptible	
	+ ²⁾	–	+	–
PMFR21 ₂₀₀ ³⁾	88	10	3	72
PMFR11 ₂₆₉	88	0	4	0
PMFR11 ₂₈₃	3	0	68	0
AA6096 ₆₈₀	86	1	1	71
M1X9-2 ₃₂₀	98	1	1	71
M10E6-4 ₂₅₀	97	0	0	75
M11E5-2 ₁₂₀₀	92	5	0	74

¹⁾ One-hundred-and-sixty-three individuals were examined for PMFR11₂₆₉ and PMFR11₂₈₃, 159 for AA6096₆₈₀, 171 for M1X9-2₃₂₀, 172 for M10E6-4₂₅₀ and 171 for M11E5-2₁₂₀₀ markers.

²⁾ + and – indicate presence and absence of the DNA marker.

³⁾ PMFR21₂₀₀, PMFR11₂₆₉ and PMFR11₂₈₃ are SCAR markers described by Sugita *et al.* (2004).

types in a segregation population even if the virus resistance gene is linked to an undesirable trait. Furthermore, conversion of these markers to specific primer-based markers makes MAS easier.

Development of the DNA markers linked to the pungency trait

An AFLP marker, M12E2-6 (720 bp) was closely linked to *C* at a distance of 4.0 cM. A CAPS marker was mapped at 0.4 cM from *C* with an interspecific F_2 population between *C. annuum* and *C. frutescens* by Blum *et al.* (2002), and this CAPS marker was developed using the clone obtained from the subtracted library of cDNA. The sequence was just the same as the reported sequence of the fibrillin gene (No. X77290) in the database (Blum *et al.* 2002). In the present study, we also attempted to map the fibrillin gene using the sequence in the database in order to secure DNA markers closer to the *C*. However, no polymorphism was found in this region between the parents. Comparing the sequencing data in the region of the fibrillin gene (renamed PAP gene) revealed the polymorphism in the microsatellite region, located 900 bp upstream from the start codon of the gene. The pungent parent, 'AC2258' had 25 repeats of TA, while non-pungent, 'K9-11' had 23 repeats at this microsatellite. Then this microsatellite was named PAP-SSR.

When linkage analysis was performed with PAP-SSR using the primers labeled with fluorescent chemicals and the *C* locus obtained from evaluating pungency, the PAP-SSR was located at the distance of 0.6 cM from *C*. The usefulness

of PAP-SSR was examined with 28 accessions of three species in *Capsicum*. Most of the accessions were homozygous, but some of them were heterozygous in this microsatellite. As many as nine alleles were found within *C. annuum*. In addition, additional allele was found in *C. chinense* (Table 4).

Nucleotide sequences of PAP-SSR fragments were analyzed on 23 accessions of three species in *Capsicum* (Fig. 4). 'Jalapeño' had 36 repeats of TA at this microsatellite locus, 'Kyouusuzu' had 27, 'K9-11', 'Miogi', 'Shousuke', 'California Wonder', 'Spirit', 'Special', 'Fiesta', 'Suizan', 'Leila' and 'Berumasari' had 25, 'AC2258' had 23, 'Criollo de Morelos 334', 'Fushimiamanaga', 'Takanotsume' and 'Kyouunami' had eleven, LS2341 and PI260429 had ten, 'Habanero', 'Miemidori' and 'Shishitou' had eight, and PI159236 had five repeats. Furthermore, 'Miemidori', 'Shishitou', 'Habanero' and PI159236 had, respectively, four bp insertions of TTTT, TTTT, TGTT and ATTT with the exception of the microsatellite. 'Miemidori' and 'Shishitou' had seven bp deletions of AATAATT. PI159236 and PI260429 had a point transition of G from T or T from A. Many alleles were found in this microsatellite region. Since some useful traits, such as disease resistance, are often found in various pungent wild species, the SSR marker obtained in the present study will be very useful in pepper breeding.

Table 4. Genotypes of PAP-SSR loci in 28 accessions of three species in *Capsicum*, using the primers labeled with fluorescent chemicals

Accessions	Species	Country of origin	Pungency	Genotype of SSR loci
K9-11	<i>Capsicum annuum</i>	Japan	non-pungent	151/151
AC2258	<i>C. annuum</i>	Mexico	pungent	147/147
F ₁ (K9-11 × AC2258)	<i>C. annuum</i>	Japan	pungent	147/151
Kyouyutaka	<i>C. annuum</i>	Japan	non-pungent	149/155
Kyouusuzu	<i>C. annuum</i>	Japan	non-pungent	155/155
Tosahikari-D	<i>C. annuum</i>	Japan	non-pungent	121/151
Shousuke	<i>C. annuum</i>	Japan	non-pungent	151/151
Miogi	<i>C. annuum</i>	Japan	non-pungent	151/151
Himukamidori	<i>C. annuum</i>	Japan	non-pungent	121/151
Kyouunami	<i>C. annuum</i>	Japan	non-pungent	122/122
Berumasari	<i>C. annuum</i>	Japan	non-pungent	151/151
Miemidori	<i>C. annuum</i>	Japan	non-pungent	115/115
Takanotsume	<i>C. annuum</i>	Japan	pungent	121/121
Fushimiamanaga	<i>C. annuum</i>	Japan	non-pungent	121/121
Nanzenji	<i>C. annuum</i>	Japan	pungent	121/151
Shishitou	<i>C. annuum</i>	Japan	pungent	115/115
Wonder bell	<i>C. annuum</i>	Japan	non-pungent	151/151
California Wonder	<i>C. annuum</i>	USA	non-pungent	151/151
Leila	<i>C. annuum</i>	EU	non-pungent	151/151
Special	<i>C. annuum</i>	EU	non-pungent	151/151
Spirit	<i>C. annuum</i>	EU	non-pungent	151/151
Suizan	<i>C. annuum</i>	EU	non-pungent	151/151
Habanero	<i>C. chinense</i>	Mexico	pungent	120/120
Criollo de Morelos 334	<i>C. annuum</i>	Mexico	pungent	121/121
Jalapeño	<i>C. annuum</i>	Guatemala	pungent	176/176
LS 2341	<i>C. annuum</i>	Malaysia	pungent	120/120
PI 159236	<i>C. chinense</i>	Peru	pungent	116/116
PI 260429	<i>C. chacoense</i>	Argentina	pungent	120/120

Fig. 4. Nucleotide sequences of PAP-SSR fragments on 23 accessions of three species in *Capsicum*. The microsatellite region located 900 bp upstream from the start codon of the fibrillin gene (Accession No.X77290). Black borders indicate the positions of SSR primers used in this study. Hyphen indicates area with lacking parts. Asterisk indicates area with coincidental parts.

Further studies will focus on 1) converting DNA markers linked to *L3* into SCAR markers, and 2) developing DNA markers linked to resistance for phytophthora blight and immature fruit colors.

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