Development of microsatellite markers in cultivated and wild species of sections *Cepa* and *Phyllodolon* in *Allium*

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Abstract The potential of microsatellite markers for use in genetic studies has been evaluated in *Allium* cultivated species (*Allium cepa*, *A. fistulosum*) and its allied species (*A. altaicum*, *A. galanthum*, *A. oscaninii*, *A. roylei*, *A. vavilovii*). A total of 77 polymerase chain reaction (PCR) primer pairs were employed, 76 of which amplified a single product or several products in either of the species. The 29 AMS primer pairs derived from *A. cepa* and 47 microsatellites primer pairs from *A. fistulosum* revealed a lot of polymorphic amplicons between seven *Allium* species. Some of the microsatellite markers were effective not only for identifying an intraspecific F_1 hybrid between shallot and bulbonion but also for applying to segregation analyses in its F_2 population. All of the microsatellite markers can be used for interspecific taxonomic analyses among two cultivated and four wild species of sections *Cepa* and *Phyllodolon* in *Allium*. Generally, our data support the results obtained from recently performed analyses using molecular and morphological markers. However, the phylogeny of *A. roylei*, a threatened species with several favorable genes, was still ambiguous due to its different positions in each dendrogram generated from the two primer sets originated from *A. cepa* and *A. fistulosum*.

Keywords Allium, microsatellite markers, DNA polymorphism

Introduction

The abundance, characterization, and the usefulness of SSR markers have been reviewed in cultivated *Allium* species (McCallum, 2007). A number of microsatellites have been identified and characterized in bulb onion, *Allium cepa* Common onion group (Fischer and Bachmann 2000) and bunching onion, *Allium fistulosum* (Song et al. 2004; Tsukazaki et al. 2007). The previous studies implied that such microsatellites could be applied as markers for several kinds of genetic analyses in a wide range of species in sections *Cepa* and *Phyllodolon* of *Allium*. In fact, a number of bunching onion microsatellites could be applicable for the purity determination of F_1 seeds as well as variety identification (Tsukazaki et al. 2006). Tsukazaki et al. (2009) demonstrated an SSR-tagged breeding scheme for *A. fistulosum*. Furthermore, many bulb onion microsatellites flanking primer pairs proved to be functional not only in shallot (*A. cepa* Aggregatum group) but also in bunching onion (Masuzaki et al. 2006). On the other hand, section *Cepa* of *Allium* includes several wild species, i.e. *Allium galanthum*, *Allium oschaninii*, *Allium vavilovii,* etc. as well as the two cultivated crops (bulb onion, shallot) (Vvedensky 1944; Havey 1995). With two wild species, *Allium altaicum* and *Allium microbulbum,* another cultivated crop, bunching onion was placed at section *Phyllodolon* (Vvedensky 1944). Bulb onion microsatellites had been applied for

assessing interspecific relatedness within these species (Fischer and Bachmann 2000). Some of the microsatellite markers can be used for interspecific taxonomic analyses among close relatives of bulb onion. There has this far been no example applied to other species by extending bunching onion microsatellites.

Allium roylei has been known as a potential gene reservoir for introducing several disease resistances to bulb onions (Kik 2002) and shows a quite unique position in the taxonomy of genus *Allium* (Fritsch and Friesen 2002). This wild species crosses easily with *A. cepa* and *A. fistulosum*, and shares a high degree of genetic similarity with other species in the two sections mentioned above. Nonetheless, most morphological characters differ remarkably from others in these sections and are much more similar to those of section *Oreiprason* (Fritsch and Friesen 2002). Further recent evidence indicates that *A. roylei* might have a hybrid origin, as its nuclear DNA profile is related to species of the sections *Cepa* and *Phyllodolon* but its chloroplast DNA profile to the section *Schoenoprasum* (van Raamsdonk et al. 1997, 2000).

In the present study, the microsatellite marker analyses using several species and their hybrids were performed with the following objectives: (1) development of microsatellite markers useful for marker-assisted selection approach using alien genes in the cultivated species of sections *Cepa* and *Phyllodolon*; (2) evaluation of heritability

via the simple test of a F_1 hybrid and the segregation analysis of its F_2 population; (3) verification of genome organization in *A. roylei* which is an insufficiently known wild species in its phylogenesis.

Materials and Methods

Plant materials

Doubled haploid line of shallot (*Allium cepa* L. Aggregatum group, 'DHA', genomes AA), bulb onion (*A. cepa* L. Common onion group, 'Hayatama-2', CC), an F_1 intraspecific hybrid between shallot and bulb onion ('DHA' × 'Hayatama-1', AC), Japanese bunching onion (*Allium fistulosum* L., 'Kujo-hoso', FF), *Allium altaicum* Pall. (I: '97010-25', II: '97213-15'), *Allium galanthum* Kar. et Kir. (I: '97205-22', II: '97205-42'), *Allium roylei* Stearn (I: '95001-2', II: '95001-6'), and *Allium vavilovii* M. Pop. et Vved. (I: '97202-8', II: '97202-2') were used for detecting intra- and interspecific polymorphisms. 'DHA' was obtained via the chromosome doubling of a gynogenetic haploid from diploid shallot 'Chiang Mai'. Its F_2 population with 38 plants was employed to examine allelic relationships at some SSR loci.

DNA extraction and PCR amplification

Total genomic DNA was isolated from fresh leaf tissue using a mini-prep DNA-isolation method (van Heusden et al. 2000) except for shallot, bulb onion, and their F_1 intraspecific hybrids, whose total genomic DNAs were extracted from the leaves according to the procedure of Dubouzet et al. (1996). Thirty microsatellite primer sets (AMS01 to AMS30; GenBank accession numbers, AJ391666 to AJ391725) for bulb onion developed by Fischer and Backmann (2000), 46 microsatellite primer pairs (bunching onion microsatellite, BMS) derived from SSR-enriched DNA libraries of Japanese bunching onion reported by Wako et al. (2002), Song et al. (2004), and Tsukazaki et al. (2007), and one onion EST-derived SSR primer pairs (Kuhl et al. 2004) were examined. PCR amplifications for the AMS primer sets were carried out according to the procedure of Masuzaki et al. (2006). For the microsatellite primer sets from bunching onion, PCR mixture was made with 0.8 μM of each of the primers, 0.2 mM dNTPs, 1 × r*Taq* buffer, 0.5 U r*Taq* polymerase (Takara, Shiga, Japan), and 100 ng template DNA in a volume of 20 μL. PCR temperatures were performed with the minor modifications of the procedure of Ohara et al. (2005). PCRs were done in a program thermal cycler (iCycler; Bio-Rad, Hercules, USA), in which the ramp times were carried out in the default conditions that adjusted temperatures at the maximum ramp

rate with the minimum ramp time. PCR products were electrophoresed and separated on 2% (w/v) agarose gel. If the PCR products were monomorphic on agarose gel, they were subjected to denaturing polyacrylamide gel electrophoresis (PAGE) with silver staining. The amplified fragments from 100 to 700 bp were scored in each *Allium* species. The markers were designated according to the primer name and the molecular size of each band in base pairs.

Phylogenetic analysis

The microsatellite fragments obtained in seven species expect for F_1 intraspecific hybrids between shallot and bulb onion were scored as either present or absent. The genetic distances were calculated according to the procedure of Nei and Li (1979) by using a program Restdist (Program to compute distance matrix from restriction sites or fragments) in software PHYLIP, version 3.68 (Felsenstein 1989). Phylogenetic analyses were performed using the Neighbor Joining Method (Saitou and Nei 1987). The graphic output of the phylogenetic trees was created with the MEGA version 4 program (Tamura et al. 2007)

Results

Polymorphisms between *Allium* species

The 29 AMS primer pairs, in which the primer pairs for AMS11 amplified no bands in all the investigated plants, and the 47 bunching onion microsatellites, or BMS, primer pairs, (including one onion EST-SSR primer pair, ACM071) revealed a lot of polymorphic amplicons between seven *Allium* species (Figs. 1 and 2; Suppl. Tables 1 and 2). A total of 782 bands were obtained by the 28 AMS primer pairs, and a total of 393 bands by 47 BMS primer pairs. The microsatellite markers of *A. fistulosum* were obtained in 26 AMS and 46 BMS primer pairs; bulb onion, 28 and 24; shallot, 28 and 28; *A. altaicum*, 26 in I / 24 in II and 38 in I / 43 in II; *A. galanthum*, 25 in I / 22 in II and 26 in I / 15 in II; *A. roylei*, 26 in I / 25 in II and 19 in I / 23 in II; *A. vavilovii*, 28 in I / II and 17 in I / 13 in II (Suppl. Tables 1 and 2). The average number ± standard deviation of polymorphic bands per AMS and bunching onion microsatellites primer were, respectively, 26.1 ± 25.8 (0 in AMS11 to 125 in AMS05) and 8.4 ± 4.9 (1 in three primer sets to 21 in AFC02E08) across the investigated plants (Fig. 2).

Evaluation of heritability of microsatellite markers in F_1 and F_2 between shallot and onion

Twenty-eight AMS primer pairs except for AMS11 and AMS24 amplified the fragments in F_1 intraspecific hybrid, which generated by crossing bulb onion with shallot, as well as the two species. Of the 28 AMS primer pairs, some microsatellite markers yielded in AMS03, AMS17, and AMS21 exhibited significant heritabilities on denaturing PAGE with silver staining (Fig. 3a, Table 1). The segregation of these markers was found to fit the monogenic segregation of 1 : 2 : 1 (Fig. 4). In addition, amplicons were observed in 23 out of the 47 bunching onion microsatellites primer pairs. Of the 23 primer pairs, only ACE127 produced heritable two microsatellite markers, i.e. ACE127-260 and -276 in *A. cepa* (Fig. 3b).

Simultaneous segregations at three loci were observed as shown in Table 2. Three different simultaneous segregations were tested for independence by chi-square tests. None of the data showed a significant difference from the expectation of independent segregation (Table 2), suggesting that these three loci are located on different chromosomes or distantly on the same chromosome. The chromosomal location of the AMS17 locus could be understood since one marker, AMS17-270, at same locus was already assigned to the chromosome 1 of *A. cepa* in our previous study

(Masuzaki et al. 2006).

Verification of genome organization in *Allium roylei*

The relationships among the entries are shown by the phyllograms in Fig. 4 on the basis of genetic distances calculated by using polymorphic amplicons between the seven *Allium* species (143 sites for AMS primer pairs and 382 sites for BMS primer pairs). The phyllogram of BMS primer pairs (Fig. 4b) showed two major nodes consisting of (I) the three species of the section *Cepa* (*A. vavilovii*, *A. cepa*, *A. galanthum*) and *A. roylei* as well as (II) the two species of the section *Phyllodolon* (*A. fistulosum* and *A. altaicum*), in agreement with one promising previous report (van Raamsdonk et al 2000). However, the results of the AMS primer pairs were different from the ones of the BMS primer pairs in clustering of *A. roylei*. While, *A. roylei* was clustered in one neutral node together with *A. galanthum* out of three different nodes (Fig. 4a). Other two nodes consisted separately of the sections *Cepa* and *Phyllodolon*.

Discussion

The most conspicuous feature of *Allium* nuclear genomes is their great size, which has made *Allium* crops complicates most molecular genetic analysis. For instance,

bulb onion nuclear genome contains 17.9 pg or 15,290 Mbp of DNA per 1C nucleus, and making it one of the hugest genomes among cultivated plants (Havey 2002). Bunching onion shows a 28 % smaller genome size than bulb onion (Ricroch et al. 2005). There seems to be a big difference between these species in not only genome size but also its structure. Jones and Rees (1968) and Narayan (1988) observed that all eight bivalents were asymmetric in interspecific hybrids between the two species. This indicated that genomic DNA differences were spread across all eight chromosomes. Chiasmata localized near the centromere of bunching onion could be explained if this region were largely euchromatic and therefore gene-rich (Havey 2002). The integration of linkage and physical maps in bunching onion showed that recombination predominantly occurs in the proximal half of chromosome arms and that 57.9% of *Pst*I/*Mse*I AFLP markers are located in close proximity to the centromeric region (Khrustaleva et al. 2005). These results suggested the presence of genes in this region. Tsukazaki et al. (2008) constructed a chromosome-specific linkage map of bunching onion based on the SSR markers used in this study. Linkage groups on same chromosome were split into two parties except a few cases, suggesting the presence of repetitive sequences (e.g. microsatellites) in the distal half of each chromosome arms. On the other hand, in bulb onion two different medium-density intraspecific and

interspecific linkage maps have thus far been developed based on several sorts of DNA markers (RFLP, SSR, SNP, InDel) (Martin et al. 2005) and *Eco*R1/*Mse*1 AFLP markers (van Heusden et al. 2000), respectively. The integration of linkage map from two different species would be possible by plotting a number of common codominant markers, such as our promising microsatellite markers with heritability and leads us to reveal a trace of the chromosomal structural changes occurred in their speciation process.

Allium roylei seems to be a potential gene reservoir for bunching onion as well as for bulb onion (Kik 2002). This wild species shows combined resistant to Botrytis leaf blight (*Botrytis squamosa*) (Lacy and Lorbeer 2008) and downy mildew (*Peronospora destructor*) (Schwartz 2008) in bulb onion. For the downy mildew, a resistance locus had been anchored to the distal end of chromosome 3R in *A. roylei* and then was successfully introduced into a bulb onion cultivar via the use of a conventional backcrossing procedure (Scholten et al. 2007). Nowadays many peoples in *Allium* research community are very interested in the origin or taxonomic position of *A. roylei*. However, one *A. roylei* strain was introduced into the European research scene in the 1960s. All living plants investigated in the world are derived from this single fertile strain (Fritsch and Friesen 2002). Sharma and Gohil (2003) had recently exploited a

small threatened population of *A. roylei* from the Bani region of Jammu province in India and observed the meiosis of PMCs in ten plants equally from two populations of *A. roylei*. The results revealed multivalent associations involving the entire complements of 16 chromosomes. The subsequent observation of somatic chromosomes in same populations clarified the extensive karyotypic variability due to repeated interchanges involving a number of non-homologous chromosomes (Sharma and Gohil 2003). The evaluation of its genetic diversity by microsatellite polymorphisms would provide informative parameters not only to carry out a hazard prediction for its threatened status but also to enforce both *in situ* and *ex situ* conservation strategies for this threatened species.

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Table 1. Segregation patterns of three AMS primers in F_2 progeny

Primer[z]	Genotype observed[y]				x^2	Probability in x^2–test
(shallot/onion)	*a*	: *ab*	: *b*	obscurity		for 1 : 2 : 1
AMS03 (196/204)	8	19	9	2	0.263	.900–.750
AMS17 (282/268)	9	18	9	2	0.105	.950–.900
AMS21 (284/277)	8	19	11	0	0.476	.900–.750

[z] Arabic numerals in parenthesis show fragment size.

[y] Allele *a* is derived from shallot, and *b* from bulb onion.

Table 2. Estimation of linkage between microsatellite loci derived from AMS primers

Loci testd for linkage	Type of freq.	Observed numbers and expected numbers (below) of genotypes										No. of plants	x^2–test for independence	d.f.	Prob.
		aa.aa	aa.ab	aa.bb	ab.aa	ab.ab	ab.bb	bb.aa	bb.ab	bb.bb	obscurity				
AMS17/AMS03	Obs.	1	6	2	2	8	6	5	3	1	4	34	7.941	4	.100–.050
	Exp.	2.125	4.25	2.125	4.25	8.5	4.25	2.125	4.25	2.125					
AMS17/AMS21	Obs.	2	5	2	4	8	6	1	5	3	2	36	0.889	4	.950–.900
	Exp.	2.25	4.5	2.25	4.5	9	4.5	2.25	4.5	2.25					
AMS03/AMS21	Obs.	3	3	3	3	13	2	2	2	5	2	36	9.222	4	.100–.050
	Exp.	2.25	4.5	2.25	4.5	9	4.5	2.25	4.5	2.25					

Figure legends

Figure 1. Amplification profiles of the primer set for AMS03 (**a**) and AFB02C01 (**b**) in *Allium* species. M, molecular size marker (100 bp DNA Ladder); CC, bulb onion; AA, shallot; FF, *A*. *fistulosum*; RR, *A*. *roylei*; GG, *A*. *galanthum*; LL, *A*. *altaicum*; VV, *A*. *vavilovii*.

Figure 2. Number distribution of 30 AMS primer sets (■), and 46 primer sets (bunching onion microsatellite, BMS) derived from SSR-enriched DNA libraries of *Allium fistulosum* and one onion EST-derived SSR primer pair (□) among the number of microsatellite markers.

Figure 3. Evaluation of heritability for microsatellite markers (**a**, AMS03-196, -204 and -214; **b**, ACE127-240, -260 and -276) in F_1 hybrid between shallot and bulb onion. M, molecular size marker (100 bp DNA Ladder); AA, shallot; CC, bulb onion; AC, F_1 intraspecific hybrid between shallot and bulb onion.

Figure 4. Segregation of F_2 population which originated from a single F_1 plant between shallot and bulb onion. S, shallot; B, Bulb onion; M, molecular size marker (100 bp DNA Ladder). An upper arrow indicates AMS03-204. A lower arrow indicates AMS03-196.

Figure 5. Phylograms generated by Neighbor Joining Method analyses of microsatellite markers amplified using 30 AMS primer sets (**a**), and 46 BMS primer sets (**b**) in seven *Allium* species. A scale of genetic distances is shown.

Figure 1, Araki et al.

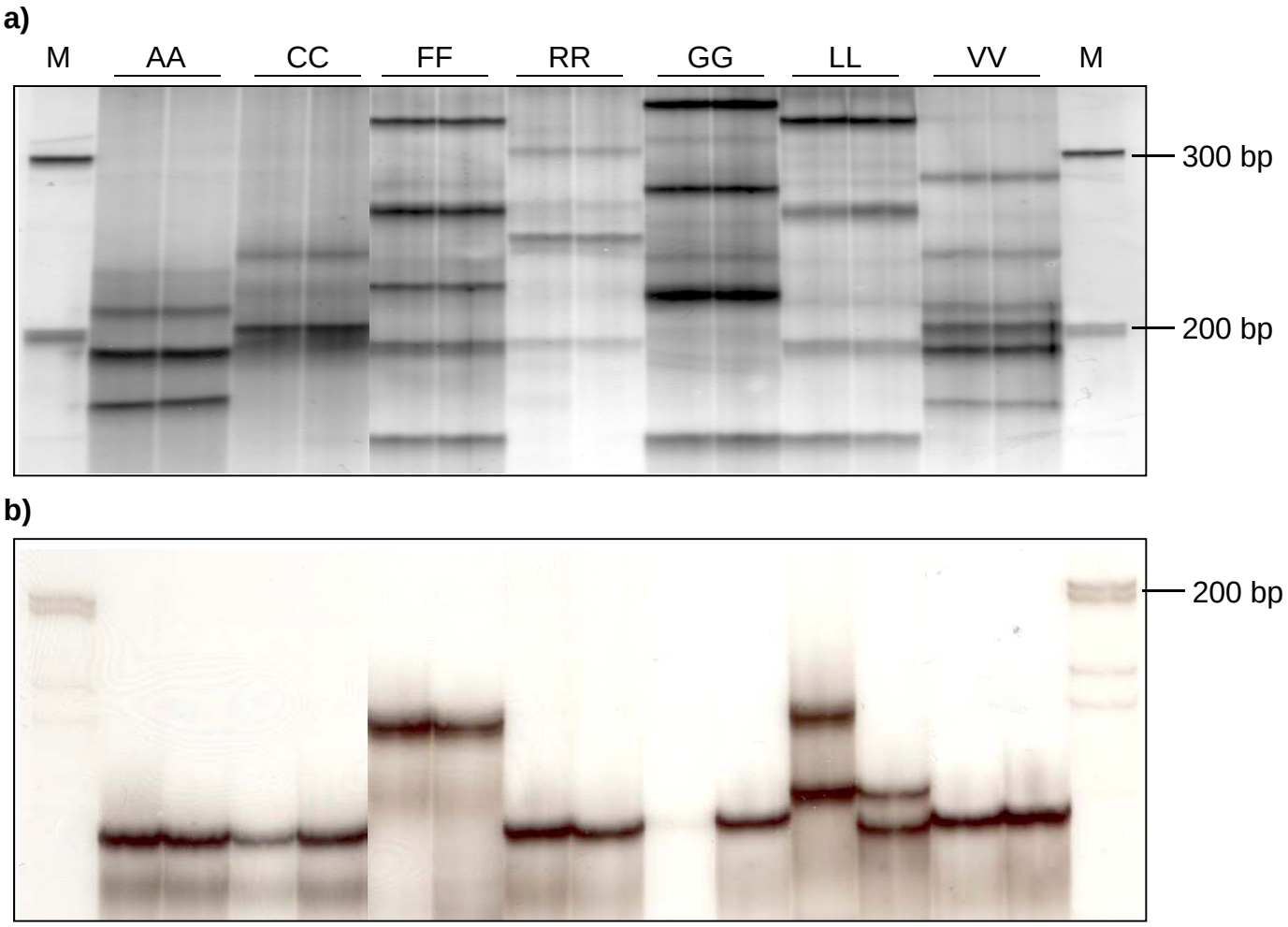


Figure 2, Araki et al.

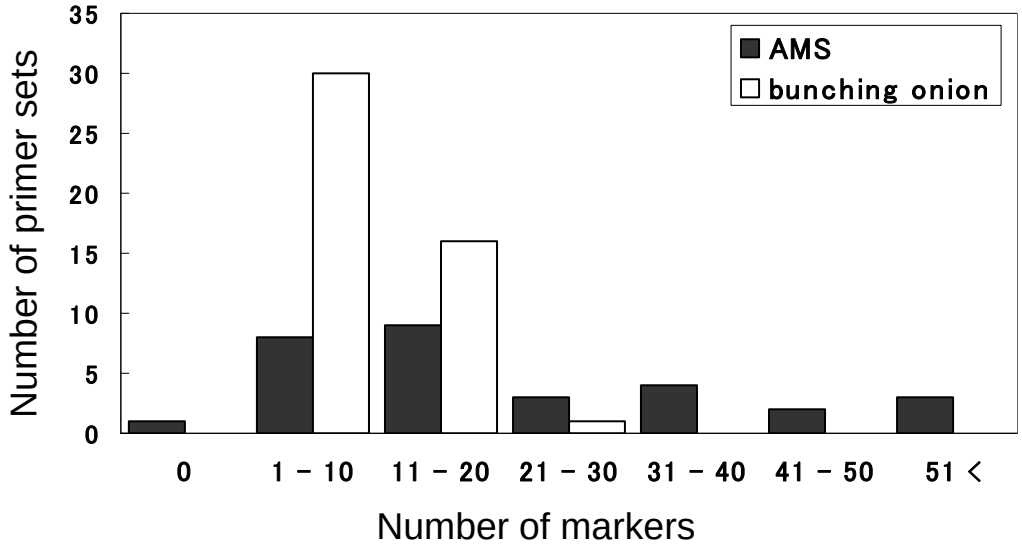


Figure 3, Araki et al.

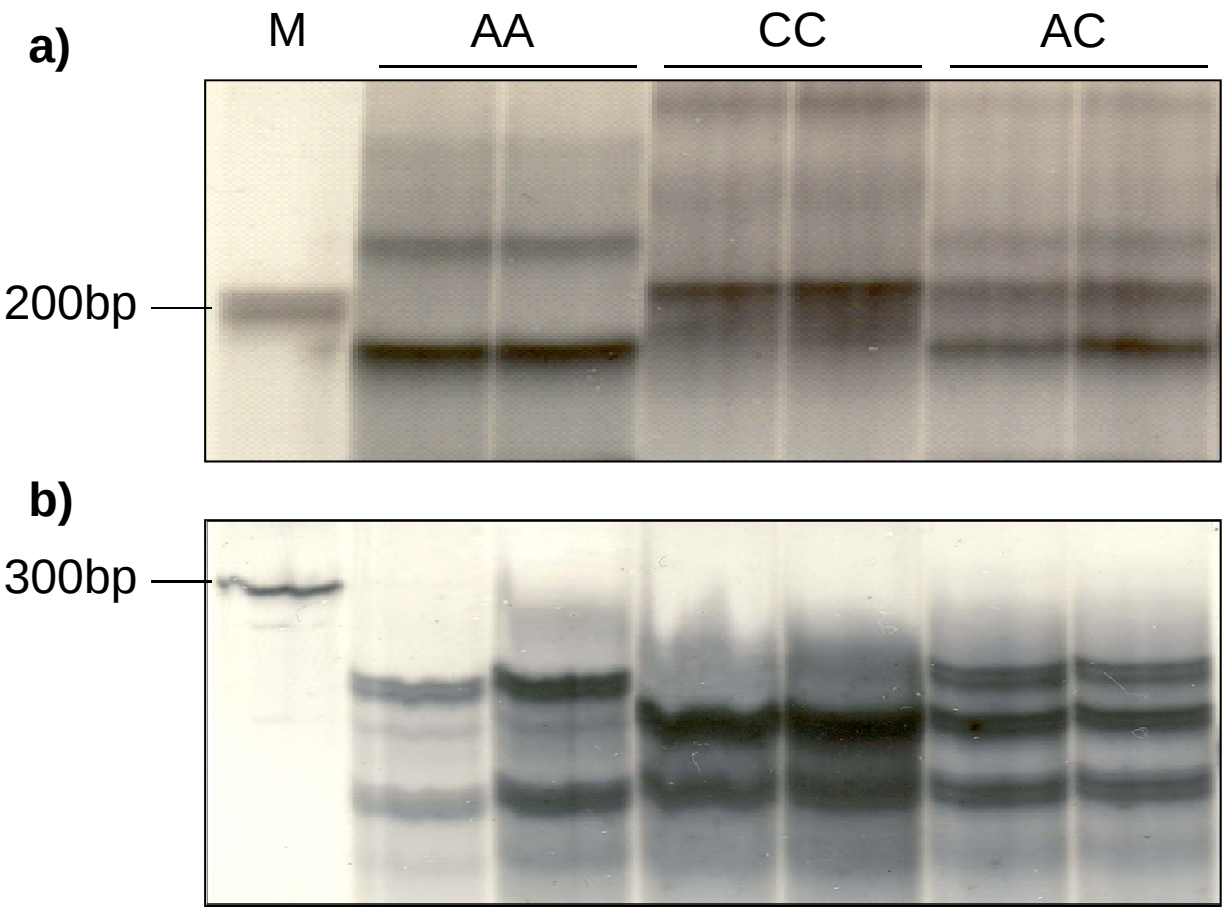


Figure 4, Araki et al.

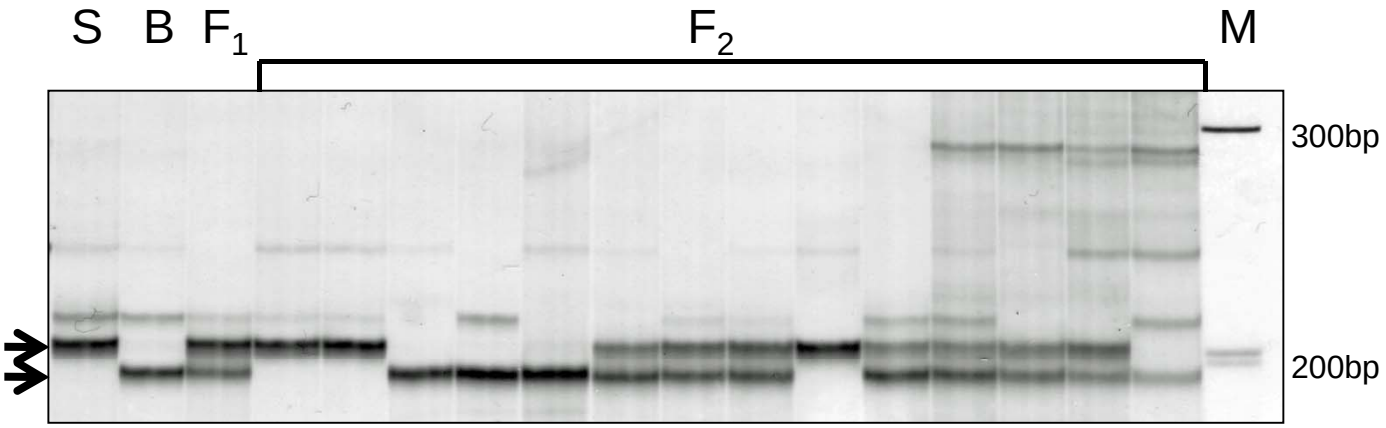
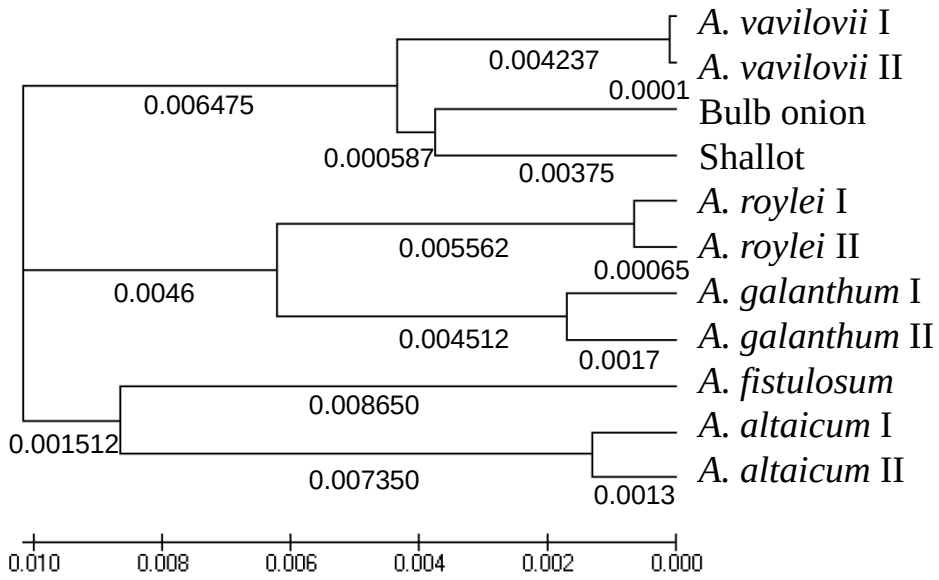
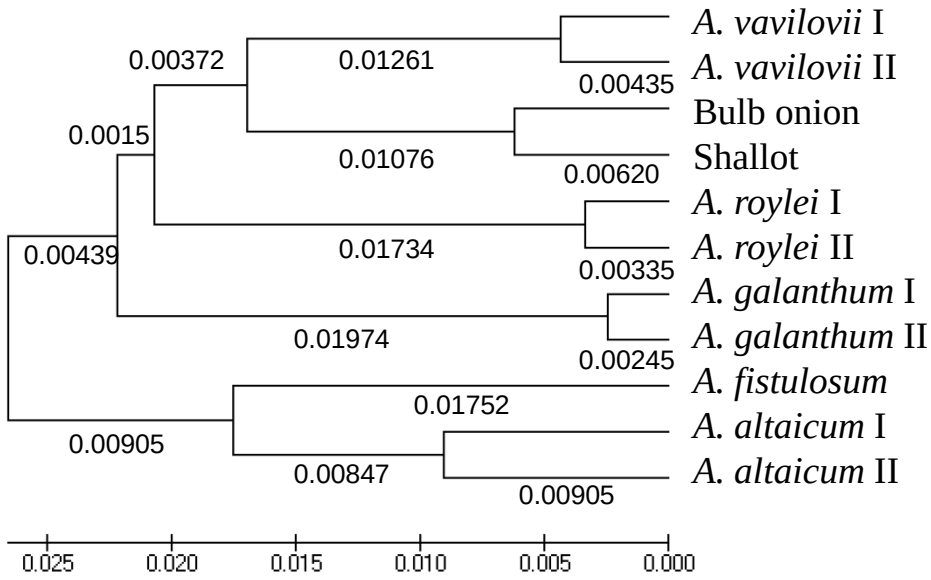


Figure 5, Araki et al.

a)



b)



Suppl. Table 1. Fragment sizes amplified from *Allium* species by microsatellite primer sets (AMS01 – 30) developed by Fischer and Bachmann (2000).

AMS[z]	Observed size (bp)[y]											
	A. fistulosum	*A. cepa*			*A. altaicum*		*A. galanthum*		*A. roylei*		*A. vavilovii*	
		onion	shallot	F_1[x]	I‘97010-25’	II‘97213-15’	I‘97205-22’	II‘97205-42’	I‘95001-2’	II‘95001-6’	I‘97202-8’	II‘97202-2’
01	130, 149, 155	149, 183, 185, 187	149, 185, 187	149, 185, 187	130, 149	130, 149	130, 164, 173	130, 164, 173	164, 173, 187	164, 173, 187	142, 164, 173, 187, 191	142, 164, 173, 187, 191
02	-	538, 560	540	540	403, 427, 440, 460, 491	-	-	-	-	-	460, 540	540
03	174, 196, 197, 228, 271, 339, 395, 427, 448, 536, 540, 573, 625	204, 244, 412, 436, 463, 478, 589	184, 196, 214, 412, 436, 463, 478, 489, 506, 589, 675	184, 196, 204, 214, 244, 412, 436, 463, 478, 489, 589	174, 197, 271, 339, 444, 463, 515, 536, 553, 606, 669	174, 197, 271, 339, 444, 463, 515, 536, 553, 606, 669	127, 174, 221, 244, 282, 319, 361, 395, 444, 478, 515, 553, 606, 653	127, 174, 221, 244, 282, 319, 361, 395, 444, 478, 515, 553, 606, 653	197, 255, 271, 307, 412, 427	197, 255, 271, 307, 412, 427	183, 196, 204, 214, 244, 288, 411, 417, 463, 480, 491, 573, 663, 691, 697	183, 196, 204, 214, 244, 288, 411, 417, 463, 480, 491, 573, 663, 691, 697
04	205, 214	205, 214	205, 214	205, 214	205, 214, 457, 469	205, 214, 457, 469	153, 158, 205, 214, 469, 480	153, 158, 205, 214, 469, 480	205, 214	205, 214	196, 199, 205, 214, 469, 480	196, 199, 205, 214, 469, 480
05	138, 158,	136,	136,	138,	136, 170,	136, 189,	119, 136,	119, 129,	114, 122,	114, 136,	113, 136,	113, 124,

184,	196,	145,	145,	154,	182,	189,	196,	214,	150,	161,	136,	150,	136,	154,	150,	154,	156,	163,	136,	174,
208,	225,	163,	160,	163,	196,	210,	225,	247,	167,	173,	170,	182,	180,	188,	157,	173,	174,	189,	189,	191,
241,	261,	176,	168,	180,	225,	247,	265,	268,	189,	196,	189,	196,	196,	206,	188,	196,	194,	196,	225,	232,
275,	284,	180,	180,	196,	272,	281,	281,	289,	210,	222,	210,	222,	218,	232,	250,	286,	225,	232,	240,	257,
300,	327,	196,	187,	204,	289,	320,	320,	333,	244,	276,	244,	276,	257,	266,	312,	336,	243,	251,	275,	289,
347,	367,	225,	194,	225,	357,	372,	372,	380,	293,	317,	293,	317,	286,	321,	372,	400,	257,	289,	307,	320,
380,	403,	241,	204,	241,	380,	410,	410,	441,	347,	372,	347,	372,	331,	348,	427,	485,	300,	320,	336,	350,
441,	469,	256,	225,	256,	441,	485,	485, 536		380,	388,	388,	410,	372,	406,	570, 663		336,	350,	372,	393,
485,	536,	294,	241,	294,	536				410,	426,	426,	441,	427,	485,			372,	393,	441,	477,
620, 663		307,	254,	300,					471,	485,	524, 620		570, 663				441,	460,	570, 650	
		322,	270,	347,					620								469,	477,		
		343,	285,	380,													520,	580,		
		367,	295,	393,													650			
		380,	343,	419,																
		393,	380,	441,																
		436,	393,	485,																
		471,	400,	570,																
		516,	436,	580,																
		570,	445,	620,																
		663	459,	663																
			471,																	
			485,																	
			516,																	
			532,																	
			570,																	
			638																	

06	-		216, 289	216, 289	216, 286	-		-		209		209		209, 279	253,	209, 279	253,	216, 289		216, 289	
07	109, 175, 567	121, 293,	109, 121, 175, 293, 362, 382, 480, 567	109, 121, 175, 293, 362, 382, 480, 567	109, 121, 175, 293, 362, 382, 480, 567	109, 175, 567	121, 293,	109, 175, 567	121, 293,	109, 175, 351, 447	121, 293,	109, 175, 351, 447	121, 293,	109, 175, 351, 480	121, 293,	109, 175, 480	121, 293,	109, 293, 567	121, 480,	109, 293, 567	121, 480,
08	181, 337, 497, 538, 563, 669	328, 356, 508, 553, 655,	196, 209, 216, 356, 638	196, 198	196, 198, 356	181		-		-		-		457, 634	617,	356		196, 198		196, 198	
09	222, 240		240, 260	222, 240, 259, 268, 482, 631	240, 260, 280, 393	222, 259, 406, 542	240, 393,	222, 259	240,	-		240, 406	393,	240, 393, 625	265, 416,	240, 393	265,	240, 393, 406	275,	240, 406	393,
10	125, 149, 164, 228,	145, 161, 216, 256,	145, 149, 151, 156,	145, 149, 151, 156,	145, 149, 151, 156,	226, 256, 357, 538,	228, 265, 365, 560,	226, 256, 357, 538,	228, 265, 365, 560,	145, 226, 256, 491,	149, 228, 265, 505,	145, 226, 256, 491,	149, 228, 265, 505,	145, 216, 228, 265,	149, 226, 256, 268,	145, 216, 228, 265,	149, 226, 256, 268,	145, 153, 170, 293,	149, 166, 284, 448,	145, 153, 170, 293,	149, 166, 284, 448,

	265, 361, 386, 393	170, 175, 256, 265, 284, 293, 312, 327, 448, 460, 476, 487, 538, 560	256, 265, 284, 293, 312, 327, 448, 460, 476, 487, 538, 560	226, 228, 256, 265, 284, 293, 312, 327, 448, 460, 476, 487, 538, 560	650, 668	650, 668	560, 571	560, 571	271, 460, 476, 560, 571, 600, 625, 650	271, 460, 476, 560, 571, 600, 625, 650	460, 476, 487, 538, 560	460, 476, 487, 538, 560
11	-	-	-	-	-	-	-	-	-	-	-	-
12	270, 304, 309, 344, 353, 360, 441, 470, 497, 520, 598, 623	255, 279, 339, 391, 460, 470	198, 255, 279	255, 279, 339, 391, 460, 470	279, 291, 486, 497, 623	279, 344, 623	195, 242	195, 242	198, 249, 250	198, 249, 250	255, 258, 279, 391	255, 258, 279, 391
13	-	103, 108, 114, 120, 160,	105, 116, 169	103, 116, 126, 132, 165,	-	-	-	-	-	-	103, 108, 120, 169, 185	103, 108, 120, 169, 185

			185		169															
14	155, 164		105, 116, 124, 141, 176, 460	105, 116, 141, 265, 270, 277, 301, 484, 563	105, 116, 124, 141, 176, 191, 351	152, 173, 270, 412	163, 189,	152, 173, 234	163, 189,	141, 270, 550, 618	173, 436,	141, 265, 550, 563	173, 478,	133, 141, 171, 234		133, 141, 171, 234		105, 116, 141, 241, 410, 484		105, 116, 141, 163, 241, 286, 362, 444	116, 163, 295, 424,
15	129, 219, 260, 278, 379, 454, 469, 500, 650	190, 224, 270, 362, 411, 461, 489, 627,	179, 181, 188, 190, 263, 278, 340, 356, 388, 394, 422, 447, 492, 500, 550, 597,	176, 184, 190, 193, 225, 245, 270, 278, 285, 291, 300, 305, 340, 356, 394, 422,	108, 188, 219, 227, 232, 278, 340, 351, 358, 381, 388, 394, 447, 459, 470, 492,	129, 218, 260, 394, 597, 686	190, 223, 270, 461, 600,	129, 218, 255, 270, 394, 461, 597, 627, 686	190, 223, 260, 358, 434, 475, 600,	112, 223, 340, 534, 650	124, 229, 411, 563,	112, 223, 340, 534, 650	124, 229, 411, 563,	171, 320, 531, 534, 563, 569	176, 461,	171, 447, 531, 563, 650	176, 461, 534, 569,	190, 215, 223, 285, 411, 516, 597, 686	193, 218, 225, 393, 447, 588,	190, 255, 351, 411, 516, 597, 600	193, 278, 393, 447, 588,

			600	447, 463, 477, 492, 500, 550, 597, 600, 627, 650, 668	500, 522, 597																
16	116, 223, 258, 441	217, 234, 281,	129, 140, 168, 169, 179, 185, 190, 244, 258, 261, 277	129, 142, 168, 258, 266, 271	129, 134, 142, 146, 154, 168, 244, 258, 266, 271	116, 230, 243, 281	223, 239, 258,	116, 230, 243, 281	223, 239, 258,	113, 126, 140, 234, 243, 258	119, 134, 168, 239,	113, 126, 140, 234, 243, 258	119, 134, 168, 239,	119, 136, 234, 258	124, 140, 242,	119, 136, 234, 258	124, 140, 242,	129, 165, 179, 188, 239, 266, 277	142, 168, 181, 230, 258, 271,	129, 165, 179, 188, 239, 266, 277	142, 168, 181, 230, 258, 271,
17	459, 559, 572	541,	109, 271, 277, 360,	274, 282, 360, 367	184, 187, 268, 274,	-		184, 192, 556, 654	187, 195, 572,	184, 190, 195, 227,	187, 192, 222, 509,	184, 190, 195, 227,	187, 192, 222, 509,	184, 195, 222, 334,	187, 197, 227, 343,	184, 195, 222, 334,	187, 197, 227, 343,	184, 268, 360, 367	187, 274,	184, 268, 360, 367	187, 274,

		367		282, 360, 367			524	524	429, 441, 537	537		
18	169, 179, 182, 197, 198, 253	171	171	171	171, 179, 182, 196, 197	179, 182, 196, 197	171, 179, 182, 204, 239, 245	171, 179, 182, 198, 204, 239, 245	171, 179, 182	171, 179, 182	171	171
19	134, 497, 573, 580	130, 134, 141, 144, 149, 187, 191, 367, 377, 420, 430	130, 134, 141, 367, 377, 420, 430, 443, 457	130, 134, 367, 377	134, 680	134, 680	750	750	425, 429	-	134	134
20	242, 352, 365, 554	224, 235, 242, 342, 357, 365, 375, 383,	145, 152, 167, 224, 235, 242, 350, 354,	152, 167, 224, 242, 352, 357, 365, 375,	235, 242, 352, 365, 375	235, 242, 357, 365, 383, 390	242, 352, 365, 383	242, 352, 365, 383	242, 352, 357, 365, 383, 390	235, 242, 352, 357, 365, 383, 390	152, 167, 216, 224, 335, 357, 365, 375, 390	152, 167, 216, 224, 335, 365

		390, 423, 436, 448, 481	365, 377, 390, 414, 448	383, 390, 448															
21	232, 236	232, 236, 245, 258, 277	232, 236, 252, 284	232, 236, 252, 277, 284	225, 236	232,	225, 236	232,	232, 252	236,	232, 252	236,	232, 252, 311	236, 271,	232, 252, 311	236, 271,	232, 245, 252	236,	232, 252	245,
22	291, 375	271, 282, 321, 356	245, 271, 282, 291, 314, 344, 366	271, 282	291, 380		291, 380		284, 344, 375	294,	284, 375	294,	297, 399		297, 399		271, 297, 380	291, 375,	271, 297, 380	291, 375,
23	137, 289, 421, 572	143, 222, 237, 421, 456, 473, 489, 536, 572	113, 135, 155, 160, 222, 237, 268, 421, 456,	139, 222, 237, 371, 375, 421, 456, 473	137, 148, 228, 274, 313, 375, 572	145, 195, 259, 290, 371, 421,	126, 148, 228, 274, 313, 375, 572	145, 195, 259, 290, 371, 421,	137, 421, 536	168, 473,	137, 421, 536	168, 473,	113, 137, 254, 289, 350, 411, 473, 764, 915	131, 246, 257, 339, 381, 421, 536, 800,	113, 137, 254, 289, 350, 421, 536, 800, 915	131, 246, 276, 339, 381, 473, 764,	109, 222, 421, 473	140, 237, 456,	109, 222, 421, 473	140, 237, 456,

				473, 617, 629																
24	154, 396, 745	179, 736,	-	-	-	154, 398, 745	179, 736,	154, 398, 745	179, 736,	424, 745	736,	424, 745	736,	-		-		-		
25	169, 205, 213	178,	120, 178, 206, 218, 225, 258, 270	116, 120, 121, 178, 206, 218, 225, 235, 270	120, 178, 206, 218, 225, 235, 243	178, 205, 213	200,	178, 205, 213	200,	153, 178, 213	169, 205,	153, 178, 213	169, 205,	169, 205, 266	178, 213,	169, 205, 266	178, 213,	206, 222	218,	206, 222	218,
26	609, 657	614,	179, 181, 189, 205, 208	179, 181, 205, 208	179, 181, 189, 205, 208	262, 274		-		202, 558	205,	202, 205		202, 205		202, 205		179, 205, 208	181,	179, 205, 208	181,
27	234, 294, 331, 391, 421, 510,	278, 308, 368, 408, 443, 573,	211, 214, 234, 265, 281, 300,	211, 214, 234, 265, 294, 308,	308, 320, 421, 463, 480, 538,	333, 408, 573	395, 443,	333, 408, 573	395, 443,	265, 421, 683	347, 573,	265, 347, 573, 683	333, 377,	265, 368, 443, 660	288, 421, 573,	265, 368, 443, 660	288, 421, 573,	268, 333, 443, 480, 538, 640	308, 347, 463, 500, 627,	268, 333, 408, 443, 480, 538,	308, 362, 439, 463, 500, 627,

	600, 627		308, 340, 353, 406, 433, 463, 480, 538, 627	320, 340, 406, 433, 476, 489, 538, 573, 627, 683	627, 667, 683															640		
28	154, 231, 280, 353, 384, 652	157, 262, 312, 358, 398,	154, 157, 250, 254, 298, 345, 393, 485, 494, 575	154, 157, 183, 192, 212, 250, 254, 319, 371, 393, 411, 423, 460, 494, 510, 520,	154, 157, 161, 250, 254, 322, 375, 384, 393, 423, 485, 494, 510, 588, 600	154, 191, 232, 264, 280, 349, 384, 463, 530, 652	157, 212, 238, 278, 313, 358, 450, 494, 563,	154, 232, 262, 313, 393, 463, 485, 563, 652	157, 238, 264, 384, 430, 473, 494, 603,	154, 191, 212, 250, 272, 413, 575, 666	157, 200, 238, 254, 370, 450,	154, 195, 238, 254, 280, 423, 494, 652	157, 212, 250, 271, 325, 431,	154, 229, 254, 295, 384, 408	157, 250, 280, 334,	154, 238, 254, 325, 358, 494, 575, 686	157, 250, 269, 334, 423, 510, 580,	154, 223, 254, 290, 358, 438, 570, 634	157, 250, 268, 334, 423, 494, 588,	154, 250, 268, 358, 438, 488, 600	157, 254, 334, 423, 485, 588,

			540, 575, 603, 634, 672											
29	353, 694	293, 315, 327	299, 330, 353, 520, 540, 681, 694	293, 315, 327	353, 488, 490, 560	353, 488, 490, 560	424, 694	424, 596, 694	590, 681,	175, 600	179,	175, 600	179,	353, 694	353, 694
30	343, 353	343, 353	412, 633	343, 353	353, 607	353, 424, 607	499, 510	683, 697		325		325		343, 353	343, 353

[z] The AMS numbers were described by Fischer and Bachmann (2000).

[y] "-" indicates absence of amplified fragments in the species.

[x] intraspecific hybrid between shallot and bulb onion

Suppl. Table 2. Fragment sizes amplified from *Allium* species by 46 microsatellite primer sets (bunching onion microsatellite, BMS) derived from SSR-enriched DNA libraries of *Allium fistulosum* developed by Tsukazaki et al. (2007) and onion EST-derived SSR primer pair (Kuhl et al., 2004).

SSR locus[z]	Observed size (bp)[y]											
	A. fistulosum	*A. cepa*			*A. altaicum*		*A. galanthum*		*A. roylei*		*A. vavilovii*	
		onion	shallot	F_1[x]	I‘97010-25’	II‘97213-15’	I‘97205-22’	II‘97205-42’	I‘95001-2’	II‘95001-6’	I‘97202-8’	II‘97202-2’
AFA06H07	243, 270	274	274	274	274	274	-	-	270	270	-	-
AFA03F08	264, 295	218, 241, 277, 291	218, 241, 273, 286	282, 291, 400	236, 259, 264	259, 264, 320	176, 218, 236, 259, 268	176, 218, 236, 259, 268	110, 170, 236, 241, 245, 250	-	-	-
AFB04B08	151, 170	229, 411	229, 411	229, 267	149, 168	147, 174	138, 147	138, 147	149	149	-	-
AFB05H03	222, 283	211, 283	211, 283	211, 283	272, 289	272, 300	-	283	-	-	-	-
AFA01A04	273	-	-	-	286	273	-	-	-	-	-	-
AFA01H03	117, 172, 175, 179	143	143	143	100, 121, 153, 183	158	145	145	143	143	145	145
AFS012	205, 209	-	183, 353, 363, 437	198, 205	200, 205	196, 200	468	-	194, 198, 295, 321, 374, 416, 437, 458	-	-	-
AFAT04E09	282	-	-	-	-	255, 277	108, 223	-	-	179	-	-
AFB02C01	129, 173	144, 152	144, 152	144, 152	119, 158, 175	112, 119, 152, 158	-	115, 154	110, 152	110, 152	112, 154	112, 154
AFRA04D09	164	-	-	-	164, 166	156, 160	471	-	-	274, 279	-	-
AFA01H12	190, 198	164, 192	154, 164	-	164, 211	190, 198	-	-	-	168	-	-
AFS104-2	205, 225	-	174, 184, 192, 200	-	210, 220, 225	170, 178, 188, 194	182	-	-	170, 176, 250	194, 205	-
AFA02A05	268, 286	-	282, 327	-	-	277	-	-	-	273	-	-

AFC02E08	116, 168	166, 180, 184, 188	166, 180, 184, 188, 205, 225, 230, 265, 487, 600	180, 184, 188	166, 215, 245, 275	120, 168	162	162	100, 134, 148	100, 134, 148	122, 168	122, 168
AFS149	194, 205	232, 253	226	226, 253	190, 198	190, 200, 216	181, 190	181, 190	185, 194	185, 194	253	253
AFA01F03	136, 138, 145, 147, 224, 238	-	-	-	173, 187, 233	140, 149, 167, 178, 223	-	-	-	-	233	233
AFS042	150, 157, 243, 248, 261	187, 248, 444	146, 248, 444	146, 248, 444	243, 257, 274	257, 274	148, 274	148, 274	159, 252, 278	243, 252, 278	248, 444	-
AFS006	258, 308	-	-	-	308	300	308	-	-	-	-	-
AFS145	210, 229	194, 210	194, 210	196, 210	210, 229	200, 219	-	-	-	-	196, 210	196, 210
AFRA03F03	248, 373, 400, 455	150, 287, 355, 391, 418, 473	287	152, 196, 287, 391	243, 274, 400, 464	235, 243, 274, 400, 455	-	-	-	-	-	-
AFA01A08	135, 308	108, 135, 308	122	129, 133, 420, 428	261, 271, 282	133, 149, 308	-	-	-	-	-	-
AFB02C10	310	-	-	-	-	-	-	-	-	-	-	-
AFAA03F01	236, 254	308, 408	408	323, 408	232, 250	239, 254	271	-	-	-	308	-
AFA03G09	291	-	-	-	-	291	-	-	-	-	-	-
AFB02G04	162, 166, 174, 177	-	-	-	174, 177	162, 174	162	162	-	-	-	-
AFA07G06	139, 170, 185	-	-	-	214	135, 139, 148, 172,	185, 191	-	-	148, 185	-	163, 198

						191						
AFC03A09	119, 122, 178, 181	-	-	-	113, 172	122, 181	-	-	-	-	-	-
AFA02F09	290	-	-	-	-	252, 276, 295	-	-	-	-	-	-
AFAT04C08	150, 183, 187	192	188	188, 205	154, 156, 173, 177	181, 185	127, 135	-	157, 175, 177	157, 175, 177	144, 194, 196	144, 194
AFAT03F07	167, 312, 359	-	111, 132, 175	-	179	128, 142	-	-	130	130	182	-
AFS221	360	-	-	-	320, 353	320, 353	-	-	-	-	-	-
AFA03D07	280, 320	-	-	-	273, 340	273, 340	-	-	-	-	-	-
AFRT08C02	240, 260, 287, 327, 353	-	-	-	-	327, 287	-	-	-	-	-	-
AFA02C03	241	144, 232, 255, 264	255, 264, 273, 282	245, 264	236	236	227, 232, 250, 255, 264, 291	227, 232, 250, 255, 264, 291	144, 232, 255	144, 232, 255	255, 264, 268	255, 264, 268
AFRT01G11	151, 163, 167, 182, 192, 250, 265	260	184, 300	-	151, 161, 165, 245, 260	151, 161, 165, 245, 260	245	245	149, 230, 250, 270	149, 230, 250, 270	-	-
AFS123-2	244, 405, 414	405, 433, 452, 490	405, 433, 452, 481	112, 433, 452, 481	395, 424	395, 424	305, 307, 414	305, 307, 414	405, 424	405, 424	405	376, 405, 433
AFA01C07	239, 322	-	-	-	311	322	-	-	294	-	-	-
AFA06	102, 125, 187	-	-	-	-	111, 191	-	-	-	-	-	-
AFA03B03	232, 245	-	-	-	245	245, 264, 390, 430	-	-	-	-	-	-
AFAT00C07	125	-	-	-	125	-	-	-	-	-	-	-

ACC008	721	177, 292, 547, 579, 753	177, 292, 516, 547, 611, 674, 689, 753	516, 547, 579, 674, 753	-	-	-	-	292, 737	737	674, 737	-
ACE010-1	174	187	189	187	176, 222	213	183	183	178	178	183, 187	-
ACE127	248	240, 260	240, 276	240, 260, 276	212, 244	212, 244	224	224	252	252	236, 252, 264	252
ACM071	158, 163	160, 161	161	158, 161	-	-	-	-	-	-	-	-
AFAT05E04	193, 282	193, 223	191, 218, 232, 245	193	209, 291, 355	193, 209	191	191	-	198	193, 236	193, 227
AFAT05G01	158, 164, 182, 189, 310, 340	-	-	-	156, 189, 219, 281, 295	156, 295	-	-	-	-	-	-
AFRT01H05	-	407	393	407	281, 393	281	281	-	281	281	-	281

[z] The SSR loci were described by Tsukazaki et al. (2007) and Kuhl et al. (2004).

[y] "-" indicates absence of amplified fragments in the species.

[x] intraspecific hybrid between shallot and bulb onion