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Classification and identification of bunching onion (*Allium fistulosum*) varieties based on SSR markers

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We investigated the correspondence between classifications based on simple sequence repeat (SSR) markers and on morphological traits for 30 bunching onion (*Allium fistulosum* L.) varieties. We also examined applicability of an assignment test for variety identification in bunching onion. Cluster analysis based on the allele frequency data at 29 SSR loci classified most of the varieties except for 'Iwatsuki-2' into the predicted variety groups, 'Kaga', 'Senju' or 'Kujo', which were categorized based on morphological traits. Although 'Iwatsuki-2' has been regarded as a member of 'Kaga' group, molecular data suggested the variety belonging to 'Kujo' rather than 'Kaga'. In the assignment test at the individual level, 89.1% of the individuals were assigned to their original variety. When the assignment was conducted based on groups each consisting of four individuals, the percentage of correct assignments was considerably improved (99.3%). These results suggested that the assignment test approach will be useful for variety identification in allogamous bunching onions, which have large within-variety genetic diversity. On the other hand, it was also suggested that sampling of true source varieties will be fundamental to avoid misjudgment.

Key Words: *Allium fistulosum*, bunching onion, classification, SSR markers, variety identification.

Introduction

Bunching onion (*A. fistulosum* L.) is considered to have originated in northwestern China and is mainly cultivated in East Asian countries, in particular in Japan, China and Korea (Kumazawa and Katsumata 1965, Ford-Lloyd and Armstrong 1993). In Japan, bunching onion has a high annual output among fruit and vegetables, following tomato, strawberry, and cucumber (MAFF 2008). Many local varieties are adapted to various climatic conditions, and are classified into four groups—'Kaga', 'Senju', 'Kujo' and 'Yagura-Negi'—according to morphological and ecological traits (Inden and Asahira 1990).

The main breeding objectives for bunching onion are disease resistance, high yield, late bolting, high consumer qualities (*e.g.*, low pungency, high sugar content) and suitability for mechanized farming (*e.g.*, vigorous seedling growth). As for the seedling growth, we reported that F_1 between the Senju and Kujo groups showed remarkable heterosis (Ohara *et al.* 2004). Also, the genetic distances based on AFLPs showed significant correlation with the degree of heterosis over the mid-parent for each seedling trait in bunching onion (Ohara *et al.* 2005a). To exploit the genetic potential of heterosis, it is important to clarify the genetic relationships among varieties and to explore heterotic groups on the basis of variety classification using molecular markers. Haishima *et al.* (1993) reported the first phylogenetic analysis of bunching onion. They reported that groups 'Kaga' and 'Senju' were categorized separately based on a cluster analysis of genetic distance data at eight isozyme loci among 13 bunching onion varieties. Previously, we attempted a classification of 11 inbred lines with cluster analysis based on 128 AFLPs (Ohara *et al.* 2005a). This classification was in accordance with the traditional classification using morphological and ecological traits. However, these markers are less polymorphic and do not reflect genetic structure (isozyme) or are less discriminative due to dominant inheritance (AFLP).

On the other hand, SSRs are ideal DNA markers due to their simplicity, reproducibility and codominant inheritance. In the genus *Allium*, Fischer and Bachmann (2000) first reported SSR markers from bulb onion (*A. cepa* L. Common onion group) and used these for a phylogenetic analysis of *Allium* species. From large-scale sequencing of bulb onion expressed sequence tags (ESTs), hundreds of EST-derived SSR markers have also been developed (Kuhl *et al.* 2004, Martin *et al.* 2005). In our previous study, we isolated thousands of SSRs from a genomic library of bunching onion (Wako *et al.* 2002, Song *et al.* 2004, Tsukazaki *et al.* 2007),

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and some of these were used to construct a linkage map of bunching onion (Ohara *et al.* 2005b, Tsukazaki *et al.* 2008). The SSR markers, which have been mapped on a linkage map, will be useful for assessing the genetic relationships of bunching onion varieties, although no attempt has been made to use them for the variety classification of bunching onion.

Moreover, variety identification based on molecular markers also becomes important from the point of view of protection of breeder's right. The assignment test, which assigns each individual to a reference variety in which its multilocus genotype is most likely to occur (reviewed by Manel *et al.* 2005), may allow us to identify the variety in allogamous plant species (Kubik *et al.* 2001, Tommasini *et al.* 2003).

The purpose of this study was (1) comparing classifications based on allele frequency data of SSR loci and on morphological traits, and (2) verifying possibility of variety identification in bunching onion which contains high degree of within-variety diversity. In this paper, we report on the first study that attempted variety classification using SSR markers.

Materials and Methods

Plant materials

A total of 30 open-pollinated (OP) varieties of bunching onion were used in this study (Table 1). These varieties include not only representatives of three major variety groups, 'Kaga', 'Senju' and 'Kujo', but also another group 'Okunegi' and one Chinese variety. These were selected as standard varieties based on principal component analysis (PCA) of nine morphological traits in our previous study (Wako *et al.* 2009, Fig. 1). The 1st PC (contributing 47.4% to the variation) was mainly associated with length-related traits, and the 2nd PC (contributing 20.0%) was associated with width-related traits.

Two species related to bunching onion were also included: an accession of *A. altaicum* Pall. COL/KAZ/1997/NIVOT/86 and an open-pollination variety of bulb onion (*A. cepa*. L.) 'Kaizuka-Wase-Ki'. These species and bunching onion belong in section *Cepa* within *Allium*, and *A. altaicum* is considered a progenitor of *A. fistulosum* (Friesen *et al.* 1999).

Total DNA was extracted from young leaves of each plant (24 individuals/variety) according to Song *et al.* (2004).

SSR markers and PCR

Thirty-three SSR markers were used for genotyping individuals (Table 2). SSR markers were selected to be evenly distributed across each linkage group of the bunching onion map (1–4 loci per linkage group) (Tsukazaki *et al.* 2008). Of these, ACM096, ACE010 and ACE044 are bulb onion EST-derived SSR markers (Kuhl *et al.* 2004, Tsukazaki *et al.* 2008), and AMS14 is a bulb onion genomic SSR marker developed by Fischer and Bachmann (2000). All markers, except for AFS103, have been located on the bunching onion linkage map (Tsukazaki *et al.* 2008).

PCR amplification was performed according to Tsukazaki *et al.* (2008); however, forward primers were fluorescent-labeled with 6-FAM, NED, PET or VIC dyes (Applied Biosystems, CA, USA) prior to use. PCR products were loaded onto a capillary DNA sequencer (ABI3730; Applied Biosystems), and analyzed using GeneMapper ver. 3.0 software (Applied Biosystems). Some PCR fragments were purified and sequenced according to Tsukazaki *et al.* (2008).

Table 1. List of 30 bunching onion varieties and two related species used in this study

Variery no.	Group (subgroup)	Accession No.	Variety name
1	Kaga (Shimonita)	JP127028	Shimonita
2		JP133859	Aji-Ipponfuto
3		JP133921	Raitei-Shimonita
4		–	Miyanegi
5	Kaga (Kaga)	JP133844	Amarume-Ipponfuto
6		JP133872	Matsumoto-Nebukafuto
7		JP25431	Gengo
8	Kaga (Iwatsuki)	JP133914	Iwatsuki-1
9		JP127040	Jionji
10		JP133870	Iwatsuki-2
11	Senju (Kurogara)	JP133875	Yoshikura
12		JP138766	Kachinanori
13		JP133888	Choho
14	Senju (Aiguro)	JP133891	Kanehiko
15		JP133906	Tokyo-Fuyuguro-Ipponfuto
16		JP133905	Omiyakuro
17	Senju (Aigara)	JP127027	Nishida
18		JP133854	Kiyotaki
19		JP25470	Toyokawafuto
20	Kujo (Koshizu)	JP127042	Koshizu-Aigarakei
21		JP25471	Koshizu-Nebuka
22		JP25474	Koshizu
23	Kujo (Kujofuto)	JP133890	Ajiyoshi
24		JP133928	Kujofuto-1
25		JP133847	Kujofuto-2
26	Kujo (Kujohoso)	JP133886	Wakamidori
27		JP133852	Asagikei-Kujo-1
28		JP133916	Asagikei-Kujo-2
29	Okunegi	JP133877	Motoharu-Bansei
30	China	JP138782	Shokyu
31	*Allium altaicum*	JP138870	COL/KAZ/1997/NIVOT/86
32	*A. cepa*	JP25385	Kaizuka-Wase-Ki

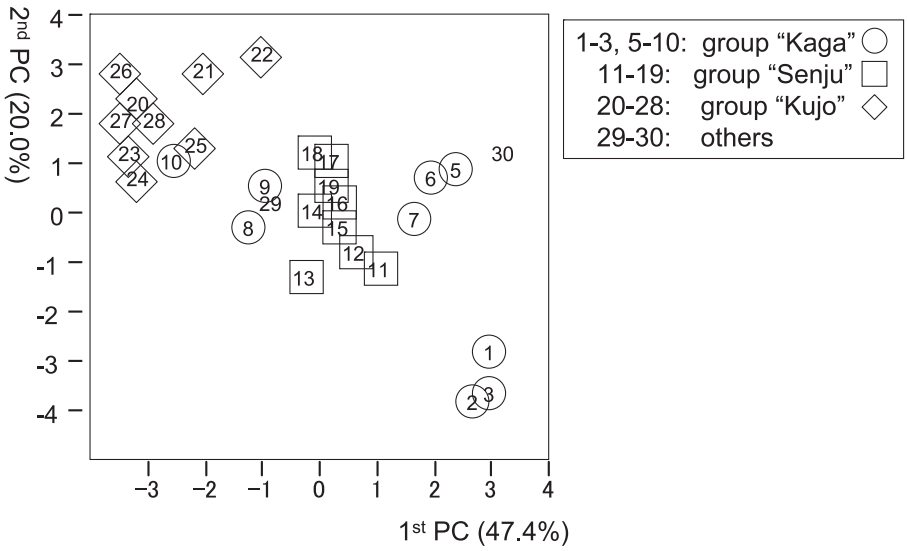

Fig. 1. Diagram of principal component analysis based on nine morphological traits (leaf blade length, leaf sheath length, flower stalk length, pedicel length, leaf blade width, leaf sheath width, flower stalk width, numbers of tillers and 1000-seed weight) of bunching onion varieties (Wako *et al.* 2009). Varieties belonging to groups 'Kaga', 'Senju' and 'Kujo' are represented by circles, squares and diamonds, respectively. Variety numbers correspond to those in Table 1.

Genotyping and statistical analysis

To assess the degree of DNA polymorphism at each SSR locus, we evaluated the number of alleles and the total heterozygosity (H_T) for each SSR locus. The H_T value was calculated according to the following formula (Anderson *et al.* 1993):

$$H_T = 1 - \sum_{i=1}^{k} P_i^2,$$

where k is the total number of alleles detected at a locus and P_i is the frequency of the ith allele in all of bunching onion individuals genotyped.

The average values for the within-variety heterogeneity (H_S), the among-variety variability (D_{ST}), and the among-variety variability relative to the total variability (G_{ST}) were calculated according to Nei (1973). The analysis of Molecular Variance (AMOVA) was calculated by using GenAlEx 6.2 (Peakall and Smouse 2006). For evaluation of genetic heterogeneity for each variety, the number of alleles, number of genotypes and proportion of individuals showing the prevailing genotype (the most frequent genotype within each variety, Pr%) were calculated. Genetic distance was calculated from allele frequencies of each locus according to Nei (1972), and cluster analysis based on both the Unweighted Pair Group Method using arithmetic Average (UPGMA) and the Neighbor-Joining (NJ) method was conducted using Populations 1.2.30beta (Langella 2007).

Assignment test

The likelihoods of multilocus genotype were calculated for each tested individual in each variety following Rannala and Mountain (1997). Briefly, we calculated the frequency for each allele of each variety by the Bayesian method. Then the likelihood of a diploid genotype occurring in a particular variety was estimated under the assumption of random mating within a variety. When the sample of the variety included a tested individual, the observed allele frequencies in the variety were obtained after removing the individual from the sample. We regarded the variety giving the highest likelihood as a variety that the tested individual was derived from.

A total of 1000 diploid genotypes were generated by resampling from each of the varieties to generate the empirical distribution of the likelihoods under the null hypothesis that a tested individual was derived from the variety following Paetkau *et al.* (2004). By comparing the observed likelihood of a tested individual with the empirical distribution in the variety, we obtained the significance level of the likelihood in the variety, which was called membership probability indicating the confidence of the tested individual to be derived from the variety. To obtain the probabilities of the tested individual to be misclassified in the wrong variety, we evaluated the relative frequency of individuals in a variety showing the membership probability of more than 5% in the assignment test to each of the varieties. The membership probabilities were calculated with GeneClass2 (Piry *et al.* 2004).

Because variety identification may be conducted in a lot unit in its practical application, we also computed the likelihood of each group, which consisted of randomly selected four individuals within each source variety, occurring in each reference variety. This assignment was conducted for 1000 groups per variety. The likelihood, L, was calculated as follows:

$$L = \frac{m!}{m_1!m_2!\cdots m_k!} P_1^{m1} P_2^{m2} \cdots P_k^{m_k}$$

where m is the total number of individuals to be assigned, m_k is the number of copies of genotype k in the to-be-assigned sample, and P_k is the frequency of genotype k in the

Table 2. Primer sequences used in this study

Marker name	Origin	Accession No.	Repeat regions	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)	PCR conditions (°C)[a]	Linkage group[b]	Map position (cM)	Reported by[c]
ACE010	bulb onion EST	CF436768	$(AC)_8(GC)_6tgc(GT)_4$	atgtaccacatggatgaaaaacaca	ggtagctgaagcaaatcaagcaa	55	5	17.7	Tsukazaki *et al.* 2008
ACM096	bulb onion EST	CF446191	$(CTA)_5$	gtgggcaattcacgttatgtgtgt	agttccaagcaccaaaagaagcac	55	6a	54.4	Kuhl *et al.* 2004
ACE044	bulb onion EST	CF436576	$(CGT)_7$	atgtacgcagaatctcgtcctttt	ttcaaattcttttgctgatgggttc	55	3a	0.0	Tsukazaki *et al.* 2008
AMS14	bulb onion genomic SSR	–	$(CA)_{28}(TA)_4$	cccctgagtaaattcaaaatcc	tccttagtataatttcggggtaac	50	2a	52.7	Fischer and Backmann 2000
AFA01A08	bunching onion genomic	AB499341	$(TA)_7(TG)_{14}$tatctaagggcatctgagt$(GA)_3$	agataagtgctcatggagcaaggg	acatccacagcaaacatagcaagc	55	X	22.8	Tsukazaki *et al.* 2007
AFA02F09	bunching onion genomic	AB499356	$(AT)_3(AC)_{14}(AT)_4g(TA)_3$	cctaggttcattaggggactatagga	ccaagcaccagtatctgcctttct	55	8	0.0	Tsukazaki *et al.* 2007
AFA06A08	bunching onion genomic	AB499376	$(AC)_6$atatacatacat$(AC)_{10}$atataca$(TA)_4$	cctcaggagaggggtattttggtt	cttgggaaaggcttctcttgaggt	55	7b	30.7	Tsukazaki *et al.* 2007
AFA10A08	bunching onion genomic	AB499401	$(TG)_{11}$	gtttagggcgtaaaatctaaacgct	gtgcttttgactaacctcgcatcc	55	X	46.6	Tsukazaki *et al.* 2008
AFA14G03	bunching onion genomic	AB499415	$(TG)_{12}$	ctccatgaatgggaggtcttctgt	gtttttccaaaaggattcacacgc	55	5	49.5	Tsukazaki *et al.* 2007
AFAA03F01	bunching onion genomic	AB499434	$(AC)_8$	cgactttgtttctcgctcttggtt	aaattgcacaaggctctgcgagat	55	7a	20.0	Tsukazaki *et al.* 2007
AFAT00B05	bunching onion genomic	AB499440	$(AT)_3$ctatgctaaccttctctaaagtacaac$(TA)_6$tttcgtgtatc$(TG)_{12}$	tgtccactattcacggcttactca	gatgggtctgcctcctccttattt	55	2a	140.1	Tsukazaki *et al.* 2007
AFAT05F03	bunching onion genomic	AB499456	$(CA)_4g(AT)_9$	ggtcagtgggtttatctacgaaaagg	tcaatctaaccgtcccctaatcca	55	2a	176.4	Tsukazaki *et al.* 2007
AFAT05G01	bunching onion genomic	AB499457	$(TG)_3$ctatgtgcacgtg$(TA)_4$attgc$(GT)_3$a$(TA)_3$ag$(TG)_4$cgttc $(GT)_3$atatca$(GT)_3(AT)_3$gacagatt$(TG)_3$c$(GT)_3$agtatacctgtatgttag $(TG)_4$cgtgagtgtaatttt(TG)5ttgc$(GT)_3$atagg$(TA)_4$aa$(TG)_8$	cgtgtgttgcagtgtttgtgtatg	ccactacaccgcacatccctatatc	55	8	204.2	Tsukazaki *et al.* 2007
AFB06E05	bunching onion genomic	AB499485	$(TC)_{13}$	tttgaattggcagagacaatggtg	tgtatggaggaagggctgaaagag	55	1b	99.9	Tsukazaki *et al.* 2007
AFC03G02	bunching onion genomic	AB499502	$(TCT)_9$	tcatctccctcttgtctgatgtcg	tccgatgaagttcgagtagaaggg	55	3b	116.9	Tsukazaki *et al.* 2007
AFB05H09	bunching onion genomic	AB499483	$(AG)_{17}(CG)_5$	gcgagcgtatcccctaagaagaat	ttggtccctagatttcactgcaca	55	3a	121.8	Tsukazaki *et al.* 2007
AFC08G05	bunching onion genomic	AB499503	$(GA)_3a(GAA)_{11}aa(AGA)_3$	gttaaaggcccattgggtatgaca	gaagttcgatgccttcatgtctca	55	2b	26.2	Tsukazaki *et al.* 2007
AFRA04B10	bunching onion genomic	AB499515	$(TG)_5$cagttgta$(TG)_8$cgcgtgcat$(GCGT)_2$gtgcatgc$(GT)_3$atgtgctac $(TG)_3$ca$(TG)_4$cagttgtgta$(TG)_5$catagttgta$(TG)_7$	gtgagtgtgcagttttgtgagggt	gtgcgcaggtccatctgtaaaaa	55	6b	33.1	Tsukazaki *et al.* 2007
AFRA04D09	bunching onion genomic	AB499516	$(TG)_3$tatgtgtatctaca$(TG)_3$ca$(TG)_7$tatatttatatgtatg$(TA)_3$	ctagcggggaatttatcctggttc	cgatacgcccaacttactcgactt	55	4b	26.4	Tsukazaki *et al.* 2007
AFRT01F02	bunching onion genomic	AB499523	$(CG)_3(CA)_6$	acgaggcataggtggatgaggtta	cagccaactcgtcataggaacgata	55	7a	73.0	Tsukazaki *et al.* 2007
AFS006	bunching onion genomic	AB499314	$(AC)_{13}(AT)_6$	gtgaccttatgtaggggttaggatt	tcgctccattcaaattaaaaa	55	3b	61.2	Tsukazaki *et al.* 2008
AFS015	bunching onion genomic	AB499317	$(TA)_4$tgtatg$(TA)_6$tg$(TA)_5$tgtg$(TA)_6$tg$(TA)_3$	atctcactgtccttgtacctgaaag	catcttgactttgtgatatttgtgc	50	1a	32.7	Ohara *et al.* 2005b
AFS017	bunching onion genomic	AB499318	$(TC)_5$catctctttac$(TC)_{15}$	tgaacttttatttatgcttcttcctc	atggaggacgaaggtgggtt	50	8	109.3	Ohara *et al.* 2005b
AFS039	bunching onion genomic	AB499319	$(AT)_8$	cgggtaataacggatatcataaaca	cagttgttacatgtggtatcagagc	50	6a	122.7	Ohara *et al.* 2005b
AFS088	bunching onion genomic	AB499323	$(TG)_{10}$	tatcttcgagcacggttcttcttgt	atggcttcgatgatggatagttgta	50	8	92.8	Song *et al.* 2004
AFS099	bunching onion genomic	AB499325	$(AC)_{13}(AT)_8$cacacttata$(AT)_3$	tgcccctcattaataacaacatgac	ttaatcgcattgacaaagtttattt	50	1b	53.5	Song *et al.* 2004
AFS103	bunching onion genomic	–	$(TA)_9(TG)_9$	ttttacctagatattttcgaatttca	catctttctttttcactagcttcctg	50	unmapped	–	Ohara *et al.* 2005
AFS111	bunching onion genomic	AB499330	$(AT)_3$gtgtctcta$(TG)_8$	tgtttaatggactttcaatgcctgt	gcattaaaatgaagaaatcccgaag	50	2b	0.0	Song *et al.* 2004
AFS131	bunching onion genomic	AB499333	$(AC)_8$	caacaaatcagagagaaacagatga	actgtatatttatgtatcactccatgtaaa	50	X	75.8	Ohara *et al.* 2005b
AFS140	bunching onion genomic	AB499334	$(TA)_3$ca$(AC)_{12}(AT)_6g(CA)_3$	cgtagcggatggtcacaaga	tggacctggaggcctagact	50	7b	77.7	Ohara *et al.* 2005b
AFS142	bunching onion genomic	AB499335	$(AC)_3$aa$(AC)_{11}(AT)_5gt(TA)_8$	tgagagaattaatattattggaggcctat	ataaaatgacaaccaacccatgtta	50	1a	133.3	Ohara *et al.* 2005b
AFS149	bunching onion genomic	AB499337	$(AC)_{11}(AT)_7$ttcacttgtaca$(TA)_3$	aaccaattgattacctctcatctgc	tgcggaccttccatagtctgtataa	50	4a	76.2	Tsukazaki *et al.* 2008
AFS156	bunching onion genomic	AB499338	$(AG)_{13}$	tcatatgcatgtccataatccgata	tttataaatagaccccgagcgaaaa	55	4a	146.1	Tsukazaki *et al.* 2008

[a] PCR conditions are given as annealing temperatures.

[b] According to Tsukazaki *et al.* (2008). Each arabic numeral correspond to chromosome 1F–8F in bunching onion. X shows a linkage group unknown for corresponding chromosome.

[c] See References.

reference variety sample (p_i^2 for homozygotes and $2p_ip_j$ for heterozygotes, with p_i, the frequency of allele i). In a similar way to the assignment at the individual level, we evaluated the membership probability by comparing the likelihood of a group to the distribution of likelihoods generated based on 1000 groups of each reference variety.

Results

Polymorphism of SSR loci in A. fistulosum

In *A. altaicum* or bulb onion, three SSR markers (AFAT05F03, AFS099 and AFS103) could not be amplified stably, whereas the other 30 markers could be amplified well. *A. altaicum* and bulb onion-specific alleles were detected at 21 and 19 loci, respectively (data not shown).

Within bunching onion, the number of alleles per locus varied from 3 to 30 with an average of 10.4, and the size difference of amplified alleles at each SSR locus ranged from 4 to 80 bp with an average of 26.6 bp (Table 3). Sequence analysis of PCR fragments verified that the differences in size of PCR fragments were due to SSR repeats (data not shown). Genomic SSRs, except for AFRT01F02 and AFS111, were more polymorphic than bulb onion EST-derived SSR markers (Table 3).

The total heterozygosity at 33 SSR loci in bunching onion ranged from 0.25 (ACM096) to 0.98 (AFS017) with an average of 0.71 (Table 3). The within-variety heterogeneity (H_S), the among-variety variability (D_{ST}), and the among-variety variability relative to the total variability (G_{ST}) ranged from 0.22 to 0.73 (average 0.53), 0.02 to 0.42 (average 0.17), and 0.03 to 0.47 (average 0.23), respectively (Table 3). The analysis of Molecular Variance (AMOVA) revealed that 77% of molecular variance was within varieties (variation among varieties was 23%).

Polymorphism within bunching onion varieties

The mean number of alleles per locus ranged from 2.1 to 5.3 with an average of 4.1 and that of genotypes within each variety ranged from 2.8 to 8.7 with an average of 6.4 (Table 4). The average Pr% of each variety ranged from 31 to 66 (Table 4). Among the 990 loci (30 varieties × 33 SSR loci), only 27 loci (2.7%) in nine varieties were considered to be genetically uniform (data not shown). However, ten of these were found in one variety (‘Motoharu-Bansei’, No. 29) and six in another variety (‘Koshizu’ No. 22).

Classification of bunching onion

Among the 30 bunching onion varieties, the genetic distances between each pair of varieties ranged from 0.04 to 0.77 with an average 0.29. The minimum genetic distance was found between ‘Tokyo-Fuyuguro-Ipponfuto’ (variety No. 15) and ‘Omiyakuro’ (No. 16), and the maximum value was shown between ‘Kiyotaki’ (No. 18) and ‘Shokyu’ (No. 30).

From a cluster analysis based on 30 SSR loci that could be amplified well in *A. altaicum* and *A. cepa*, bunching onion varieties were classified into three clusters. These clusters corresponded to the expected variety groups, that is, ‘Kaga’ (clusters A3 and B2), ‘Senju’ (clusters A2 and B1) and ‘Kujo’ (clusters A3 and B3). An exception was ‘Iwatsuki-2’ (No. 10), which classified as a member of the ‘Kujo’ group (Fig. 2). In contrast, two related species, *A. altaicum* and *A. cepa* , were located as outgroups (Fig. 2).

Assignment test

The 89.1% of individuals (637/715) showed the highest likelihood in their original variety (Table 5). All individuals

Table 3. Polymorphism at 33 SSR loci among 30 bunching onion varieties

Locus	Linkage group[a]	Size range (bp)	Alleles	H_T[b]	H_S[c]	D_{ST}[d]	G_{ST}[e]
AFS015	1a	316–384 (68)	13	0.93	0.60	0.33	0.36
AFS142	1a	240–253 (13)	8	0.89	0.50	0.39	0.44
AFS099	1b	210–263 (53)	19	0.96	0.68	0.27	0.29
AFB05H09	1b	266–274 (8)	5	0.53	0.42	0.11	0.20
AMS14	2a	116–164 (48)	21	0.79	0.62	0.17	0.21
AFAT00B05	2a	191–211 (20)	11	0.72	0.59	0.13	0.18
AFAT05F03	2a	213–237 (24)	13	0.82	0.69	0.13	0.16
AFS111	2b	229–233 (4)	3	0.43	0.37	0.05	0.13
AFC08G05	2b	284–305 (21)	8	0.64	0.55	0.09	0.14
ACM096	3a	270–276 (6)	3	0.39	0.29	0.10	0.25
AFC03G02	3a	153–177 (24)	8	0.60	0.49	0.10	0.17
AFS006	3b	282–308 (24)	13	0.78	0.62	0.16	0.21
AFB06E05	3b	127–155 (28)	10	0.75	0.61	0.14	0.19
AFS149	4a	191–213 (22)	12	0.74	0.57	0.17	0.23
AFS156	4a	208–222 (14)	8	0.66	0.57	0.09	0.14
AFRA04D09	4b	114–154 (40)	5	0.54	0.39	0.16	0.29
ACE010	5	165–171 (6)	4	0.49	0.35	0.13	0.28
AFA14G03	5	212–222 (10)	6	0.62	0.51	0.11	0.18
ACE044	6a	184–188 (5)	5	0.55	0.47	0.08	0.15
AFS039	6a	283–312 (29)	15	0.96	0.66	0.30	0.31
AFRA04B10	6b	284–300 (16)	7	0.77	0.57	0.20	0.26
AFAA03F01	7a	230–238 (8)	5	0.56	0.54	0.02	0.03
AFRT01F02	7a	220–224 (4)	3	0.47	0.35	0.12	0.25
AFA06A08	7b	142–198 (56)	13	0.85	0.64	0.21	0.25
AFS140	7b	191–246 (55)	18	0.97	0.66	0.30	0.31
AFA02F09	8	264–298 (34)	11	0.76	0.57	0.19	0.25
AFAT05G01	8	294–314 (20)	8	0.25	0.22	0.02	0.09
AFS017	8	189–269 (80)	19	0.98	0.68	0.30	0.30
AFS088	8	155–170 (15)	8	0.93	0.53	0.40	0.43
AFA01A08	X	296–322 (26)	14	0.84	0.73	0.10	0.12
AFA10A08	X	284–304 (20)	10	0.41	0.38	0.04	0.09
AFS131	X	154–166 (12)	7	0.89	0.47	0.42	0.47
AFS103	unmapped	198–262 (64)	30	0.97	0.73	0.24	0.25
Mean		(27)	10.4	0.71	0.53	0.17	0.24

[a] According to Tsukazaki *et al.* (2008). Each arabic numeral corresponds to chromosome 1F–8F in bunching onion. X shows a linkage group unknown for corresponding chromosome.
[b] Total heterozygosity.
[c] Within-variety heterogeneity.
[d] Variation among varieties.
[e] Relative differentiation among varieties.

Table 4. Polymorphism of 30 bunching onion varieties at 33 SSR loci

Variety no.[a]	No. of alleles			No. of genotypes			Pr%[b]		
	Min.	Max.	Average	Min.	Max.	Average	Min.	Max.	Average
1	1	7	3.2	1	16	5.2	13	100	44
2	2	11	3.9	3	13	5.8	11	87	45
3	2	10	4.1	2	18	6.8	6	88	38
4	2	10	4.1	2	16	6.5	8	96	43
5	2	10	4.6	2	15	6.9	8	83	38
6	2	9	4.1	2	13	6.0	8	83	42
7	2	9	4.1	3	14	6.4	8	96	36
8	2	13	5.3	2	16	8.7	13	96	36
9	2	8	4.0	3	15	6.8	9	83	39
10	2	10	4.8	3	15	7.7	8	88	36
11	2	8	4.6	2	15	7.1	9	88	35
12	2	9	4.4	2	15	6.5	16	96	42
13	2	11	4.5	2	18	7.2	4	96	39
14	1	7	3.7	1	11	5.5	14	100	48
15	2	10	4.7	2	14	7.4	5	83	31
16	2	12	4.5	2	17	6.8	9	92	36
17	2	10	4.2	2	15	6.1	17	96	42
18	1	7	3.8	1	11	5.5	8	100	48
19	1	9	4.4	1	15	7.1	4	100	35
20	1	8	3.4	1	13	4.8	11	100	56
21	1	11	3.6	1	15	5.4	13	100	45
22	1	5	3.0	1	11	4.6	13	100	53
23	2	7	3.9	2	15	6.4	8	86	38
24	2	8	5.0	3	15	7.6	13	75	37
25	2	10	5.1	3	14	7.7	9	71	33
26	2	11	4.5	2	16	7.5	4	75	32
27	2	8	4.2	2	15	6.8	8	75	36
28	2	7	3.9	2	13	6.4	11	88	36
29	1	5	2.1	1	8	2.8	13	100	66
30	1	9	3.8	1	16	5.8	9	100	46
Mean			4.1			6.4			41

[a] Corresponding to Table 1.

[b] Calculated as follows:
Pr% = (No. of plants with prevailing genotype)/(No. of plants genotyped) × 100.

of five varieties ('Choho', 'Nishida', 'Koshizu-Nebuka', 'Motoharu-Bansei' and 'Shokyu') showed the highest likelihood in their original variety. Most of the varieties showed a high degree of correct assignment (over 80%), except for five varieties: 'Kujofuto-2' (50.0%), 'Omiyakuro' (60.9%), 'Kujofuto-1' (66.7%), 'Tokyo-Fuyuguro-Ipponfuto' (70.8%) and 'Yoshikura' (79.2%). In 'Omiyakuro', five of 23 individuals were misassigned to 'Yoshikura'. Four of 24 individuals in 'Yoshikura' were also misassigned to 'Omiyakuro'. The genetic distance between these varieties was considerably low, at 0.05. Similar results were found in combinations with 'Tokyo-Fuyuguro-Ipponfuto' and 'Kanehiko', and 'Kujofuto-2' and 'Wakamidori', whose genetic distances were 0.04 and 0.06, respectively. In Table 6, we showed the proportion of individuals which showed the membership probability more than 0.05 in each reference variety. Most of individuals of the source varieties except for 'Shokyu' showed the membership probability more than 0.05 in multiple reference varieties. For example, more than 90% of individuals of 'Choho' showed the membership probability more than 0.05 in 'Yoshikura', 'Kachinanori', 'Tokyo-Fuyuguro-Ipponfuto' and 'Omiyakuro' as well as in itself. In contrast, 'Shokyu' showed the probability above 0.05 only in itself. As a result, the total number of varieties which was not excluded as the possible origin, ranged from one of 'Shokyu' to 17 of 'Raitei-Shimonita' and 'Miyanegi'.

When the assignment was conducted based on the group unit, the percentage of correct assignment was considerably improved (99.3%; Table 7). All groups of 23 varieties showed the highest likelihood in their original variety. Even though the percentage of correct assignment of 'Kujofuto-2' was 50.0% at the individual level, the value reached 93.9%. We showed the proportion of groups which showed the membership probability more than 0.05 in each reference variety in Table 8. The most prominent change was the membership probability of 'Asagikei-Kujo-2' to 'Kujofuto-1'; despite all individuals of 'Asagikei-Kujo-2' showed the probability more than 0.05 in 'Kujofuto-1' at the individual level, no groups showed the values above 0.05. As a result, the total number of varieties which was not excluded as the possible origin, generally decreased as compared to the assignment at the individual level. In the four varieties ('Iwatsuki-1', 'Kiyotaki', 'Motoharu-Bansei', 'Shokyu'), all groups showed the probability above 0.05 only in themselves.

Discussion

In the present study, we investigated DNA polymorphisms within and/or among bunching onion varieties based on SSR markers and attempted variety classification based on allele frequencies at each locus. Within 30 bunching onion varieties, the number of alleles per locus at each SSR loci ranges from 3 to 30 with an average 10.4 (Table 3). Compared with the polymorphisms at SSR loci reported in maize accessions and inbred lines (alleles per locus range from 2 to 13 with an average of 6.5, Labate *et al.* 2003) and in our previous study using bunching onion F_1 varieties (alleles per locus range from 2 to 7 with an average of 3.1, Tsukazaki *et al.* 2006), these results show that bunching onion varieties have a very high degree of genetic diversity. The average value of within-variety heterogeneity (H_S, 0.53) was much higher than that of the among-variety variability (D_{ST}, 0.17) in the present study (Table 3). Estimates for within-population variation obtained with SSR markers are almost three times as high as the values with dominant (RAPD, AFLP) markers. Because of the high variability of SSRs, the values for within-population variation are often much higher than that for among-population variation (reviewed in Nybom 2004).

All varieties had a high degree of heterozygosity

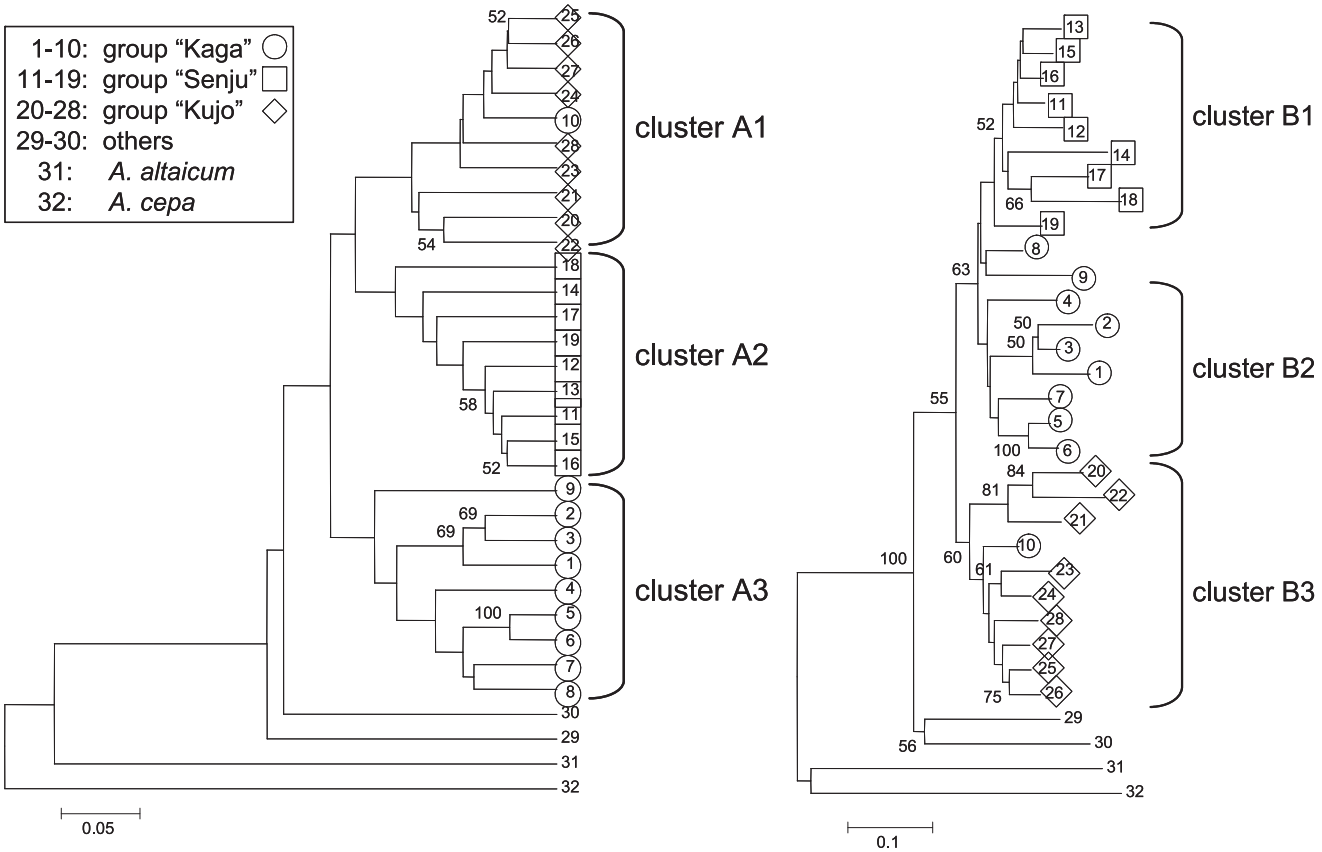

Fig. 2. Dendrogram of 30 bunching onion varieties and two related species generated by UPGMA (left) and NJ (right) cluster analysis of genetic distance based on 30 SSR loci. Varieties belonging to groups 'Kaga', 'Senju' and 'Kujo' are represented by circles, squares and diamonds, respectively. Variety numbers corresponds to those in Table 1. Bootstrap values were shown when these were above 50%.

(Table 4). However, the morphological and ecological traits are highly uniform within each variety (data not shown). Bunching onion is usually propagated by open-pollination. Therefore, this high heterogeneity at SSR loci within varieties might be held through the generations. On the other hand, nine varieties were estimated as highly uniform at between one and 10 loci (data not shown). One reason for this may be that these highly uniform SSR loci might not be tightly linked to QTLs, but may still influence growth or reproductive traits, such as flowering time or seed productivity, and expected or unexpected genetic selection for these markers may have occurred during breeding or seed production. The fixation of alleles can be also caused by random genetic drift especially when population size is small. The relationship between the SSR loci and these traits will be revealed with the accumulation of data from genomic studies in the future.

Cluster analysis was effective for variety classification (Fig. 3). The classification of bunching onion varieties was in accordance with the traditional classification based on morphological traits, for example, group 'Kaga' (Wako *et al.* 2009, Fig. 1). Similar results were obtained for variety classification based on molecular markers in bunching onion using isozymes (Haishima *et al.* 1993) and AFLPs (Ohara *et al.* 2005b). In contrast, our classification based on SSR markers will have the advantage because the SSRs are higher polymorphisms and more discriminative due to codominant inheritance than these markers. The simple nucleotide polymorphism (SNP) and insertion-deletion (InDel) markers are also informative markers. However, in bulb onion, the phylogenic analysis of SSRs was consistent with known pedigrees and previous marker evaluation, although SNPs and Indels did not reveal clear relationships among populations (Jakše *et al.* 2005). 'Iwatsuki-2' (variety No. 10) was categorized as a member of the 'Kujo' group based on cluster analysis using both UPGMA and NJ methods (Fig. 2). This variety is also located near the 'Kujo' varieties using PCA data based on nine morphological traits in our previous study (Wako *et al.* 2009, Fig. 1). These data indicate that 'Iwatsuki-2' might not belong to 'Kaga', and therefore, should be excluded from standard varieties of the 'Kaga' group as previously defined (Wako *et al.* 2009). As a landrace, 'Iwatsuki' is traditionally classified as belonging to the 'Kaga' group due to its dormancy in the winter season (Kumazawa and Katsumata 1965, Aoba 1987). However, Iwasaki (2007) reported that four 'Iwatsuki' varieties could be divided into three groups by a cultivation test, and one of these was considerably similar to a 'Kujo' variety. In addition, several 'Iwatsuki' varieties could not be explicitly distinguished from 'Kujo' varieties from PCA data based on either morphological traits or growth habits (Wako *et al.*

Table 5. Results of assignment test[a]

Source variety			Reference variety																												Correct
No.	Name	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	%
1	Shimonita	23	22		1																												95.7
2	Aji-Ipponfuto	22	1	20	1																												90.9
3	Raitei-Shimonita	24		1	22					1																							91.7
4	Miyanegi	24				23															1												95.8
5	Amarume-Ipponfuto	24					23	1																									95.8
6	Matsumoto-Nebukafuto	24					1	23																									95.8
7	Gengo	24							22	2																							91.7
8	Iwatsuki-1	24			2				1	20							1																83.3
9	Jionji	23								1	21						1																91.3
10	Iwatsuki-2	24									1	20														1		2					83.3
11	Yoshikura	24											19				1	4															79.2
12	Kachinanori	24								1			1	21			1																87.5
13	Choho	24													24																		100.0
14	Kanehiko	24														23					1												95.8
15	Tokyo-Fuyuguro-Ipponfuto	24							1				1				17	5															70.8
16	Omiyakuro	23			1								5		1		2	14															60.9
17	Nishida	24																	24														100.0
18	Kiyotaki	24											1							23													95.8
19	Toyokawafuto	24															1				23												95.8
20	Koshizu-Aigarakei	24																				23						1					95.8
21	Koshizu-Nebuka	24																					24										100.0
22	Koshizu	24																					1	23									95.8
23	Ajiyoshi	24										1													23								95.8
24	Kujofuto-1	24										2													2	16	1	2	1				66.7
25	Kujofuto-2	24										1													1	2	12	5	3				50.0
26	Wakamidori	24																									2	22					91.7
27	Asagikei-Kujo-1	24																								1			20	3			83.3
28	Asagikei-Kujo-2	24										1																	1	22			91.7
29	Motoharu-Bansei	24																													24		100.0
30	Shokyu	24																														24	100.0
	Total	715																															89.1

[a] The number of individuals which showed the highest likelihood in the reference variety is scored.

Table 6. The proportion of individuals which showed the membership probability more than 0.05 in each reference variety[a]

Source variety			Reference variety																													Total No. of varieties not excluded as origin[b]
No.	Name	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
1	Shimonita	23	**0.957**	0.348	0.696	0	0.174	0.043	0	0.696	0	0	0.174	0.522	0	0	0.261	0.130	0	0	0.130	0	0	0	0	0.087	0.043	0	0	0	0	0	13
2	Aji-Ipponfuto	22	0.091	**1.000**	0.591	0	0.091	0.045	0.091	0.682	0	0.045	0.318	0.045	0	0	0.273	0.045	0	0	0.136	0	0	0	0	0.045	0	0	0	0	0	0	14
3	Raitei-Shimonita	24	0	0.417	**0.958**	0	0.167	0.167	0.083	0.917	0.042	0.125	0.250	0.167	0	0	0.375	0.292	0	0	0.125	0	0	0	0.042	0.292	0.167	0	0.042	0	0	0	17
4	Miyanegi	24	0	0	0.042	**0.875**	0.167	0.167	0	0.333	0	0.125	0.250	0.292	0.042	0	0.458	0.375	0	0	0.208	0	0	0	0.042	0.083	0.042	0	0.042	0.042	0	0	17
5	Amarume-Ipponfuto	24	0	0	0.042	0.083	**0.875**	0.792	0.208	0.750	0	0	0.375	0.292	0.042	0	0.500	0.542	0	0	0.375	0	0	0	0	0.167	0.125	0	0	0	0	0	14
6	Matsumoto-Nebukafuto	24	0	0	0.083	0.125	0.875	**0.958**	0.250	0.917	0	0	0.375	0.167	0	0	0.625	0.417	0	0	0.250	0	0	0	0	0.125	0	0	0	0	0	0	12
7	Gengo	24	0	0	0	0	0.042	0	**0.958**	0.875	0	0.042	0.208	0.167	0	0	0.417	0.208	0	0	0.125	0	0	0	0	0.167	0.042	0	0	0.042	0	0	12
8	Iwatsuki-1	24	0	0	0.083	0	0.083	0.042	0.042	**0.792**	0	0.042	0.208	0.250	0	0	0.333	0.208	0	0	0.083	0	0	0	0	0.083	0.042	0	0	0	0	0	13
9	Jionji	23	0	0	0	0	0	0	0	0.217	**0.913**	0	0.391	0.087	0	0	0.261	0.174	0	0	0.043	0	0	0	0	0.130	0	0	0	0	0	0	8
10	Iwatsuki-2	24	0	0	0	0	0	0	0	0.292	0.042	**0.875**	0.083	0.167	0	0	0.167	0	0	0	0.083	0	0.042	0	0.208	0.833	0.667	0.292	0.333	0.125	0	0	14
11	Yoshikura	24	0	0	0	0	0	0	0	0.542	0	0	**0.958**	0.875	0.333	0	0.917	0.958	0	0	0.333	0	0	0	0	0.083	0	0	0	0	0	0	8
12	Kachinanori	24	0	0	0	0	0.042	0	0.042	0.500	0	0.125	0.917	**1.000**	0.250	0	0.792	0.833	0.042	0	0.375	0	0	0	0.042	0.167	0.083	0	0.042	0	0	0	15
13	Choho	24	0	0	0	0	0	0	0	0.542	0	0	1.000	0.917	**0.958**	0	0.917	0.958	0	0	0.500	0	0	0	0	0	0	0	0	0	0	0	7
14	Kanehiko	24	0	0	0	0	0	0.042	0.083	0.333	0	0	0.583	0.375	0	**0.917**	0.667	0.500	0	0	0.167	0	0	0	0	0	0	0	0	0	0	0	9
15	Tokyo-Fuyuguro-Ipponfuto	24	0	0	0	0	0.042	0.042	0.042	0.625	0	0	0.958	0.750	0.333	0	**1.000**	0.917	0	0	0.250	0	0	0	0	0.042	0	0	0	0	0	0	11
16	Omiyakuro	23	0	0	0.087	0	0	0	0	0.783	0.043	0.174	1.000	1.000	0.696	0	1.000	**1.000**	0.087	0	0.652	0	0	0	0	0.217	0.043	0	0	0	0	0	13
17	Nishida	24	0	0	0	0	0	0	0	0.375	0	0	0.667	0.958	0.250	0	0.667	0.792	**0.958**	0	0.292	0	0	0	0	0	0	0	0	0	0	0	8
18	Kiyotaki	24	0	0	0	0	0	0	0	0.208	0	0	0.250	0.542	0	0	0.208	0.208	0	**0.917**	0.125	0	0	0	0	0	0	0	0	0	0	0	7
19	Toyokawafuto	24	0	0	0	0.042	0	0	0.042	0.708	0	0.125	0.750	0.708	0.042	0	0.708	0.792	0	0	**1.000**	0	0	0	0	0.167	0.083	0	0.042	0	0	0	13
20	Koshizu-Aigarakei	24	0	0	0	0	0	0.042	0	0.083	0	0.917	0	0.417	0	0	0	0	0	0	0.083	**0.917**	0.250	0	0.417	1.000	0.833	0.417	0.250	0.125	0	0	13
21	Koshizu-Nebuka	24	0	0	0	0	0	0	0	0.292	0	0.625	0.042	0.167	0	0	0.083	0.042	0	0	0.042	0	**1.000**	0	0	0.667	0.500	0	0.083	0	0	0	11
22	Koshizu	24	0	0	0	0	0	0	0	0.250	0	0.875	0	0.083	0	0	0	0	0	0	0	0.542	0.750	**0.917**	0.250	0.958	0.875	0.333	0.375	0.083	0	0	12
23	Ajiyoshi	24	0	0	0	0	0	0	0	0.250	0	0.667	0	0.167	0	0	0.042	0	0	0	0.042	0	0	0	**0.958**	0.958	0.625	0.500	0.250	0.292	0	0	11
24	Kujofuto-1	24	0	0	0	0	0	0	0	0.125	0	0.542	0	0.042	0	0	0.083	0.042	0	0	0	0	0	0	0.292	**1.000**	0.625	0.292	0.417	0.125	0	0	11
25	Kujofuto-2	24	0	0	0	0	0	0	0	0.167	0	0.458	0	0.042	0	0	0	0	0	0	0.042	0	0	0	0.083	0.833	**0.875**	0.625	0.500	0.292	0	0	10
26	Wakamidori	24	0	0	0	0	0	0	0	0	0	0.667	0	0	0	0	0	0	0	0	0	0	0.042	0	0.250	1.000	0.958	**0.958**	0.458	0.125	0	0	8
27	Asagikei-Kujo-1	24	0	0	0	0	0	0	0	0.500	0	0.958	0.208	0.250	0	0	0.042	0.042	0	0	0.042	0	0.042	0	0.500	1.000	0.958	0.708	**1.000**	0.708	0	0	14
28	Asagikei-Kujo-2	24	0	0	0	0	0	0	0	0.417	0	0.833	0.083	0.250	0	0	0.083	0	0	0	0	0	0.042	0	0.292	1.000	0.792	0.458	0.833	**1.000**	0	0	12
29	Motoharu-Bansei	24	0	0	0	0	0	0	0	0	0	0	0	0.042	0	0	0	0	0	0	0	0	0	0	0.042	0.792	0.375	0	0.083	0	**0.917**	0	6
30	Shokyu	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.958	1

[a] The values in boldface represent the proportion in the case of self-assignment. The largest value other than the value to the originating variety is underlined.

[b] The total number of reference varieties that one or more individuals of the source variety showed the membership probability more than 0.05.

Table 7. Results of assignment test based on groups of four individuals[a]

Source variety			Reference variety																													Correct
No.	Name	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	%
1	Shimonita	1000	1000																														100.0
2	Aji-Ipponfuto	1000		1000																													100.0
3	Raitei-Shimonita	1000			1000																												100.0
4	Miyanegi	1000				1000																											100.0
5	Amarume-Ipponfuto	1000					1000																										100.0
6	Matsumoto-Nebukafuto	1000						1000																									100.0
7	Gengo	1000							1000																								100.0
8	Iwatsuki-1	1000								1000																							100.0
9	Jionji	1000								1	999																						99.9
10	Iwatsuki-2	1000										1000																					100.0
11	Yoshikura	1000											987				2	11															98.7
12	Kachinanori	1000											1000																				100.0
13	Choho	1000												1000																			100.0
14	Kanehiko	1000														1000																	100.0
15	Tokyo-Fuyuguro-Ipponfuto	1000															986	14															98.6
16	Omiyakuro	1000											71				24	905															90.5
17	Nishida	1000																	1000														100.0
18	Kiyotaki	1000																		1000													100.0
19	Toyokawafuto	1000																			1000												100.0
20	Koshizu-Aigarakei	1000																				1000											100.0
21	Koshizu-Nebuka	1000																					1000										100.0
22	Koshizu	1000																						1000									100.0
23	Ajiyoshi	1000																							1000								100.0
24	Kujofuto-1	1000										2														989	3	2	4				98.9
25	Kujofuto-2	1000																								5	939	55	1				93.9
26	Wakamidori	1000																										1000					100.0
27	Asagikei-Kujo-1	1000																								8	1		984	7			98.4
28	Asagikei-Kujo-2	1000																												1000			100.0
29	Motoharu-Bansei	1000																													1000		100.0
30	Shokyu	1000																														1000	100.0
	Total	30000																															99.3

[a] The number of groups which showed the highest likelihood in the reference variety is scored.

Table 8. The proportion of groups which showed the membership probability more than 0.05 in each reference variety[a]

Source variety			Reference variety																														Total No. of varieties not excluded as origin[b]
No.	Name	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
1	Shimonita	1000	**0.902**	0	0.203	0	0	0	0	0.614	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
2	Aji-Ipponfuto	1000	0	**0.873**	0.446	0	0	0	0	0.605	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
3	Raitei-Shimonita	1000	0	0	**0.842**	0	0	0	0	0.875	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
4	Miyanegi	1000	0	0	0	**0.895**	0	0	0	0.122	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
5	Amarume-Ipponfuto	1000	0	0	0	0	**0.912**	0.162	0	0.775	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
6	Matsumoto-Nebukafuto	1000	0	0	0	0	0.853	**0.906**	0	0.860	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
7	Gengo	1000	0	0	0	0	0	0	**0.879**	0.988	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
8	Iwatsuki-1	1000	0	0	0	0	0	0	0	**0.901**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
9	Jionji	1000	0	0	0	0	0	0	0	0.002	**0.726**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
10	Iwatsuki-2	1000	0	0	0	0	0	0	0	0.036	0.001	**0.898**	0	0	0	0	0	0	0	0	0	0	0	0	0	0.348	0.078	0	0	0	0	0	5
11	Yoshikura	1000	0	0	0	0	0	0	0	0.594	0	0	**0.909**	0.007	0	0	0.476	0.013	0	0	0.001	0	0	0	0	0	0	0	0	0	0	0	6
12	Kachinanori	1000	0	0	0	0	0	0	0	0.678	0.001	0.009	0.360	**0.872**	0.009	0.195	0.004	0	0	0.001	0	0	0	0	0.001	0.001	0	0	0	0	0	0	11
13	Choho	1000	0	0	0	0	0	0	0	0.420	0	0	0.030	0.014	**0.902**	0	0.092	0.001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
14	Kanehiko	1000	0	0	0	0	0	0	0	0.011	0	0	0	0	0	**0.874**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
15	Tokyo-Fuyuguro-Ipponfuto	1000	0	0	0	0	0	0	0	0.510	0	0	0.120	0	0.001	0	**0.909**	0.013	0	0	0.002	0	0	0	0	0	0	0	0	0	0	0	6
16	Omiyakuro	1000	0	0	0.004	0.003	0.006	0	0	0.976	0.207	0.026	0.988	0.417	0.425	0	0.999	**0.912**	0.002	0	0.291	0	0	0	0	0.021	0.018	0	0	0	0	0	15
17	Nishida	1000	0	0	0	0	0	0	0	0.092	0	0	0	0	0	0	0	0	**0.896**	0	0	0	0	0	0	0	0	0	0	0	0	0	2
18	Kiyotaki	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.883**	0	0	0	0	0	0	0	0	0	0	0	0	1
19	Toyokawafuto	1000	0	0	0	0	0	0	0	0.755	0	0	0	0	0	0	0	0	0	0	**0.899**	0	0	0	0	0	0	0	0	0	0	0	2
20	Koshizu-Aigarakei	1000	0	0	0	0	0	0	0	0.001	0	0.758	0	0	0	0	0	0	0	0	0	**0.900**	0	0	0	0.559	0.441	0.003	0	0	0	0	6
21	Koshizu-Nebuka	1000	0	0	0	0	0	0	0	0.420	0	0	0.030	0.014	0	0	0.092	0.001	0	0	0	0	**0.906**	0	0	0	0	0	0	0	0	0	6
22	Koshizu	1000	0	0	0	0	0	0	0	0	0	0.291	0	0	0	0	0	0	0	0	0	0	0	**0.898**	0	0.002	0.011	0	0	0	0	0	4
23	Ajiyoshi	1000	0	0	0	0	0	0	0	0.004	0	0.191	0	0	0	0	0	0	0	0	0	0	0	0	**0.906**	0.883	0.106	0	0	0	0	0	5
24	Kujofuto-1	1000	0	0	0	0	0	0	0	0.012	0	0.231	0	0	0	0	0	0	0	0	0	0	0	0	0.001	**0.893**	0.450	0.001	0.003	0	0	0	7
25	Kujofuto-2	1000	0	0	0	0	0	0	0	0.002	0	0.067	0	0	0	0	0	0	0	0	0	0	0	0	0	0.460	**0.920**	0.044	0.003	0	0	0	6
26	Wakamidori	1000	0	0	0	0	0	0	0	0	0	0.278	0	0	0	0	0	0	0	0	0	0	0	0	0	0.666	0.879	**0.916**	0	0	0	0	4
27	Asagikei-Kujo-1	1000	0	0	0	0	0	0	0	0.190	0	0.836	0	0	0	0	0	0	0	0	0	0	0	0	0	0.987	0.998	0.228	**0.919**	0	0	0	6
28	Asagikei-Kujo-2	1000	0	0	0	0	0	0	0	0.420	0	0	0.030	0.014	0	0	**0.092**	0.001	0	0	0	0	0	0	0	0	0	0	0	0.902	0	0	6
29	Motoharu-Bansei	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.898**	0	1
30	Shokyu	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.888**	1

[a] The values in boldface represent the proportion in the self-assignment.

[b] The total number of reference varieties that one or more groups of the source variety showed the membership probability more than 0.05.

2009). Clarification of the classification of 'Iwatsuki' varieties will require further investigations into their genetic backgrounds. On the other hand, two related species, *A. altaicum* and *A. cepa*, were distinctly different from all of the bunching onion varieties since these species have specific alleles at 21 and 19 loci, respectively (data not shown). It was reported that *A. fistulosum* formed subclusters with *A. altaicum* whereas it was distinct from *A. cepa* based on both RFLPs of mt and cpDNAs (Yamashita *et al.* 2001).

In bulb onion, several studies of variety classification based on RAPD markers have been reported (Wilkie *et al.* 1993, Le Thierry D'ennequin *et al.* 1997, Tanikawa *et al.* 2002). Tanikawa *et al.* (2002) reported 22 varieties that were categorized into six groups by cluster analysis. Le Thierry D'ennequin *et al.* (1997) also reported that the seed-propagated shallot is more closely related to bulb onion than to vegetatively propagated shallot. However, the chromosomal locations of the RAPD markers used in these studies have not been determined, and the relationship between clusters and various agronomic traits was also unclear. Despite the fact that some SSR-specific primers do not amplify in closely related species, SSR loci are highly preserved within species or genus. Hence, SSR markers will be a powerful tool for the classification of *Allium* species. In the present study, we evenly selected SSR markers from each linkage group of our bunching onion map (Tsukazaki *et al.* 2008), thus, our variety classification based on these anchor markers is considered to reflect genetic background of bunching onion. However, as Virk *et al.* (2000) pointed out, it is noticed that misleading information on genetic relationships could be obtained if anchor markers were selected from a linkage map of the population between closely related genotypes. Thus, it might be more appropriate to use unmapped markers for variety classification especially in crops that their genetics remain relatively unstudied using molecular-based approaches.

In the assignment test at the individual level (Table 5), the percentage of correct assignments was 89.1%, which is lower than that in an allogamous crop, perennial ryegrass (100%; Kubik *et al.* 2001) and that in a partially allogamous rape (99%; Tommasini *et al.* 2003). This relatively low value of bunching onion was mainly due to the high level of misassignment in several particular varieties. The percentages of correct assignments were 50.0%, 60.9% and 66.7% in 'Kujofuto-2', 'Omiyakuro' and 'Kujofuto-1', respectively, while most other varieties showed a relatively high percentage of correct assignment (*i.e.*, greater than 90%). Varieties with low correctness were genetically similar to several other varieties, as shown in the cluster analysis (Fig. 2). The calculation of membership probability indicated that most of individuals except for those of 'Shokyu' had several other varieties which were not excluded as the origin (Table 6). For example, although all individuals of 'Choho' showed the highest likelihood in itself (Table 5), the probabilities of membership were also above 0.05 in several other varieties such as 'Yoshikura', 'Kachinanori', 'Tokyo-Fuyuguro-Ipponfuto' and 'Omiyakuro' as well as in itself. This indicates that individuals of 'Choho' could be judged as other varieties such as above mentioned, if true source variety (that is 'Choho') is not included in a reference dataset. However, the assignment test at the group level improved the accuracy of the assignment. The percentage of correct assignment was considerably improved (99.3%; Table 7) and the total number of varieties which was not excluded as the origin decreased as compared to that at the individual level (Table 8). These results suggest that an assignment test, especially based on a group level, will be effective for variety identification in the allogamous bunching onions, which contain large amount of genetic variation within a variety. However, even in the assignment test at the group level, only four varieties ('Iwatsuki-1', 'Kiyotaki', 'Motoharu-Bansei' and 'Shokyu') were perfectly excluded from other varieties (Table 8). This suggests that sampling of true source varieties will be fundamental in variety identification in bunching onion to avoid misjudgment.

In the previous study, we proposed an "SSR-tagged breeding" scheme to enhance the efficiency, ease and accuracy of variety identification and F_1 purity testing (Tsukazaki *et al.* 2006), and demonstrated the feasibility of this scheme by using a bunching onion landrace (Tsukazaki *et al.* 2009). This scheme is especially effective for F_1 purity test with investigating the degree of uniformity of designed heterozygosity at selected SSR loci. However, it is necessary for selecting plants homozygous at several SSR loci in the foundation seed field in advance. We selected 26 double and 40 quadruple homozygotes selected from preselected individuals (108 and 147, respectively) by stand observation at stock field (Tsukazaki *et al.* 2009). Thus, the SSR-tagged breeding scheme, even with a very small number of markers, is efficient for the identification of newly bred varieties, and consequently for F_1 purity tests, in allogamous crops. In contrast, high degree of the correct assignment rate was obtained by assignment test based on groups of individuals. The assignment test approach will be useful for variety identification not only in bunching onion but also any allogamous crops. This will be also applicable for F_1 varieties. In addition, assignment tests combined with exclusion methods can have an important role especially in conservation of landraces or genetic resources. Therefore, the scheme for variety identification in allogamous crops should be selected by the purpose and situations.

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