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Note

Strawberry cultivar identification based on hypervariable SSR markers

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We genotyped strawberry cultivars by two newly selected and two previously reported SSR markers. All four markers produced interpretable electropherograms from 75 accessions consisting of 72 *Fragaria* × *ananassa* cultivars or lines and three octoploid *Fragaria* species accessions. These SSR markers were highly polymorphic; in particular, one of the newly developed markers, FxaHGA02P13, was capable of distinguishing all of the accessions except for a mutant strain that was derived from another accession in the set. When two markers were combined, all 48 full-sib individuals could be distinguished. Fingerprinting patterns were reproducible between multiple samples, including the leaves, sepals, and fruit flesh of the same accession. Principal-coordinate analysis of the 75 accessions detected several groups, which reflect taxon and breeding site. Together with other available markers, these SSR markers will contribute to the management of strawberry genetic resources and the protection of breeders' rights.

Key Words: cultivated strawberry, fingerprinting, *Fragaria* × *ananassa*, genotyping, microsatellite.

Introduction

Cultivated strawberry, *Fragaria* × *ananassa* ($2n = 8x = 56$), is an economically important crop worldwide. In Japan, the value of strawberry production was more than 168 billion yen in 2007, making strawberry the third most valuable crop after rice and tomato (Ministry of Agriculture, Forestry and Fisheries 2010; http://www.maff.go.jp/j/tokei/index.html).

Strawberry cultivars are usually vegetatively propagated from runners. Recently, the unregulated propagation and distribution of patented cultivars has become a serious problem (Kunihisa 2010). To protect breeders' rights and to maintain the genetic integrity of genetic resources, several molecular marker systems have been developed for strawberry cultivar identification. Kunihisa *et al.* (2003, 2005, 2009) developed 25 cleavage amplified polymorphic sequence (CAPS) markers and confirmed their ability to distinguish among 117 cultivars, except for mutant strains derived from others in the set. This system is highly reproducible, produces results that are simple to interpret, and does not require an autosequencer for the detection of polymorphisms. Meanwhile, because the degree of polymorphism of each marker was low, a number of markers were required for cultivar identification. In addition, CAPS marker analysis requires the use of restriction enzyme digestions. For these reasons, analyses of large numbers of samples can be time-consuming. Tasaki *et al.* (2008) developed three sets of multiplex PCR primers based on RAPD (random amplified polymorphic DNA)-STS (sequence tagged site) markers. A combination of the three sets could distinguish 25 cultivars, though the number of cultivars distinguished by any individual primer set was only 1 to 4. Because the authors analyzed only 25 cultivars, it was unknown whether the marker system could distinguish additional cultivars.

Recently, the number of strawberry cultivars registered in Japan has substantially increased. Whereas only 35 cultivars were registered from 1980 to 1989, 53 were registered from 1990 to 1999, 120 were registered from 2000 to March 2011, and applications for registration have been published for 20 more. To ensure the sensitive and rapid identification of the increasing number of cultivars, we expect that additional polymorphic markers will be also useful.

In general, simple sequence repeats (SSRs) are highly polymorphic, reproducible, and easy to assay; for these reasons, they have been widely used for cultivar identification in clonally propagated crops such as grape, apple, pear, potato, carnation, turfgrass species, and others (Ashkenazi *et al.* 2001, Bowers *et al.* 1996, Guilford *et al.* 1997, Kimura *et al.* 2002, 2009, Smulders *et al.* 2003, Wang *et al.* 2010, Yamamoto *et al.* 2006). Also for strawberry, development of SSR markers suitable for cultivar identification is a current

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area of interest. Shimomura and Hirashima (2006) developed four SSR markers for strawberry, but the degree of polymorphism of those markers was relatively low; even when the most polymorphic marker was used, 2 of the 10 cultivars tested showed the same genotype and could not be distinguished. Govan *et al.* (2008) selected 10 SSR markers suitable for the fingerprinting of strawberry cultivars, and Brunings *et al.* (2010) tested the applicability of these 10 markers for the genotyping of Florida strawberry cultivars. Brunings *et al.* (2010) found that two markers, EMFv104 and EMFvi136, were both capable of distinguishing all accessions when combined with one additional marker. In these studies (Brunings *et al.* 2010, Govan *et al.* 2008), 'Nyoho' was the only Japanese cultivar analyzed, so the applicability of those markers for discriminating Japanese cultivars was not determined.

In this study, we revealed the fingerprinting patterns of 75 *Fragaria* accessions, including a number of Japanese cultivars, by using two newly developed SSR markers (T. Nunome *et al.*, manuscript in preparation) and two SSR markers (EMFv104 and EMFvi136) reported in previous studies (Brunings *et al.* 2010, Govan *et al.* 2008). We also evaluated the discrimination power of these markers for highly related individuals by analyzing a set of 48 full-sibs. By using the detected SSR polymorphisms among the 75 accessions, we analyzed the genetic relationships within this group. This study is one of the first to identify highly polymorphic SSR markers suitable for the identification of Japanese strawberry cultivars.

Materials and Methods

We collected leaves from 75 accessions consisting of 72 *F.* × *ananassa* cultivars or lines and three octoploid *Fragaria* species accessions (Table 1), which were grown at the National Agricultural Research Center for Tohoku Region. For most of the accessions, we collected leaves from multiple plants to confirm reproducibility. For four cultivars ('Miyazaki-natsuharuka', 'Natsuakari', 'Summer-berry' and 'Summer-princess'), we also collected sepal and fruit flesh samples to confirm the reproducibility of the genotype when different tissue samples were used. To estimate the discriminating power of these SSR markers for highly related individuals, we also collected leaves from 48 full-sib individuals produced from a cross between 'Sagahonoka' and 'Summerberry'. Total DNA was extracted by using a modified PEG method (Rowland and Nguyen 1993) with Plant DNAzol Reagent (Invitrogen, Carlsbad, CA, USA), as described by Sugimoto *et al.* (2005), or by using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Each of the samples was genotyped by using four SSR primer pairs (Table 2). The primers for EMFv104 and EMFvi136 were previously reported by Govan *et al.* (2008). FxaHGA02P13 and FxaAGA21F11 were selected from 200 primer pairs, which were newly developed by Nunome *et al.* (in preparation), according to the ease of PCR amplification and interpretation, reproducibility and degree of polymorphism. For each sample, the polymerase chain reaction (PCR) was performed by two methods to examine the robustness of the fingerprint patterns: the original method of Schuelke (2000) and a slightly modified method based on the method of Schuelke (2000) (hereafter, "modified method"). In the original method (Schuelke 2000), the PCR reaction mix contained 5.0 μL of GoTaq Colorless Master Mix (Promega, Madison, WI, USA), 1.6 pmol of the reverse primer, 1.6 pmol of the universal fluorescent (D2, D3, or D4)-labeled M13(-21) primer, 0.4 pmol of the forward primer with a M13(-21) tail at its 5′ end, and 10 ng template DNA in a total volume of 10 μL. A leader sequence (GTTTCTT) was appended to the 5′ end of the reverse primers to minimize the appearance of stutter bands in the electropherograms (Brownstein *et al.* 1996). Thermocycling conditions were as follows: 5 min at 94°C; then 30 cycles of 30 s at 94°C, 45 s at 56°C and 45 s at 72°C; followed by 8 cycles of 30 s at 94°C, 45 s at 53°C and 45 s at 72°C and a final extension at 72°C for 10 min. In the modified method, the fluorescent-labeled primer was added after the first PCR amplification, which was performed in a 10-μL volume. The amplification was performed as follows: 5 min at 94°C, followed by 10 cycles of 45 s at 94°C, 1 min at 52°C and 45 s at 72°C. Then, 0.8 μL of 2 pmol/μL of the fluorescent-labeled primer was added to each PCR tube. The second amplification was performed as follows: 15 s at 94°C; 30 cycles of 45 s at 94°C, 1 min at 45°C and 45 s at 72°C and a final extension step at 72°C for 7 min. These PCR reactions were carried out in a GeneAmp PCR System Model 9700 (Applied Biosystems, Foster City, CA, USA) or a PCR Thermal Cycler Dice (Takara, Tokyo, Japan). The PCR products were run on a CEQ 8000 autosequencer (Beckman-Coulter, Fullerton, CA, USA).

We analyzed the relationships between accessions based on the genotypes of the four SSRs in the following way. For each accession, any peak detected in at least one accession was scored as present (1) or absent (0) across all of the SSR markers. Then, the binary data were combined into a single matrix and used for the calculation of a genetic similarity matrix with the aid of PAUP 4.0 (Swofford 2002). Based on the matrix, we conducted a principal-coordinate (PCO) analysis to visualize the relationships among the accessions by using the software PCO 1.0 (Iwata 2004). For marker EMFvi136, we used the genotype data obtained by the modified method, because more peaks could be detected (see Results and Discussion).

Results and Discussion

In this study, we genotyped strawberry cultivars by two newly selected SSR markers and two previously reported SSRs (Brunings *et al.* 2010, Govan *et al.* 2008). All four markers revealed interpretable electropherograms from all accessions surveyed. The genotype of each accession is listed in Supplemental Table 1. When we performed genotyping by

Table 1. Name and country of origin of strawberry accessions analyzed in this study

Accession No.	Accession name	Country	Accession No.	Accession name	Country
1	Aiko	USA	39	Morioka 32	Japan
2	Akihime	Japan	40	Morioka 33	Japan
3	Akitaberry	Japan	41	Morioka 34	Japan
4	Amaotome	Japan	42	Morioka 35	Japan
5	Benihoppe	Japan	43	Natsuakari	Japan
6	Beruruju	Japan	44	Nohime	Japan
7	Blakemore	USA	45	Nyoho	Japan
8	Bolero	UK	46	Ohishi-shikinari	Japan
9	Cambridge Favourite	UK	47	Otomegokoro	Japan
10	Cardinal	USA	48	Oze-akarin	Japan
11	Dekoruju	Japan	49	Pajaro	USA
12	Deutch Evern	Germany	50	Pechka	Japan
13	Donner	USA	51	Pegasus	UK
14	Elsanta	Netherlands	52	Pelican	USA
15	Everberry	Japan	53	Raiho	Japan
16	Florence	UK	54	Red Gauntlet	UK
17	Fukuba	Japan	55	Reiko	Japan
18	Harumi	Japan	56	Sachinoka	Japan
19	Haruyoi	Japan	57	Sagahonoka	Japan
20	Hatsukuni	Japan	58	Sequoia	USA
21	Hecker	USA	59	Summer-berry	Japan
22	Hitachihime	Japan	60	Summer-candy	Japan
23	Hogyoku	Japan	61	Summer-drop	Japan
24	Hokowase	Japan	62	Summer-fairy	Japan
25	HS 138	Japan	63	Summer-princess	Japan
26	Ibarakiss	Japan	64	Summer-tiara	Japan
27	Kaorino	Japan	65	Tioga	USA
28	Karenberry	Japan	66	Tochihime	Japan
29	Kitaekubo	Japan	67	Tochihitomi	Japan
30	Kitanokagayaki	Japan	68	Tochiotome	Japan
31	Komachiberry	Japan	69	Toyonoka	Japan
32	Mae	UK	70	Tsuburoman	Japan
33	Marshall	USA	71	Uzushio	Japan
34	Miyazaki-natsuharuka	Japan	72	Yayoihime	Japan
35	Miyoshi	Japan	73	*Fragaria chiloensis* 'PI551445'	
36	Moikko	Japan	74	*F. virginiana* #1[a]	
37	Morioka 16	Japan	75	*F. virginiana* #2[b]	
38	Morioka 30	Japan			

[a] Seeds were purchased from B&T World Seeds, Paguignan, France, in 2002.

[b] Germplasm conserved in the National Agricultural Research Center for Tohoku Region, Morioka, Japan.

the original PCR amplification method of Schuelke (2000), the number of peaks scored per marker ranged from 20 (FxaAGA21F11) to 34 (EMFv104) and the number of unique genotypes ranged from 61 (FxaAGA21F11) to 74 (FxaHGA02P13; Table 2). When we performed genotyping by the modified method, two additional peaks (145 and 155) were detected at EMFvi136. The fingerprinting patterns detected by FxaHGA02P13, FxaAGA21F11 and EMFv104, did not change, suggesting that these three SSR markers are more robust and less susceptible to the difference in PCR method than EMFvi136.

One of the newly developed markers, FxaHGA02P13, was capable of distinguishing 74 unique genotypes among 75 accessions (Table 2). Thus, FxaHGA02P13 has high discriminating ability comparable to that of EMFv104, which showed the highest discriminating ability in previous studies (Brunings *et al.* 2010, Govan *et al.* 2008). 'Akitaberry', a somatic mutant that originated from 'Morioka 16', had the identical genotypes to that of 'Morioka 16' across all four SSR markers, as was seen in the previous CAPS analysis (Kunihisa *et al.* 2009). Although the number of unique genotypes detected by FxaAGA21F11 (61 among 75 accessions, i.e., 81.3%) was lowest among the four markers surveyed here, the discriminating rates for 10 markers selected from over 100 SSRs by Govan *et al.* (2008) ranged from 83.3% to 100.0% in their study, and were much lower in Florida cultivars (Brunings *et al.* 2010), indicating that FxaAGA21F11 has discriminating power comparable to those previously selected markers. Although a comprehensive analysis using multiple markers is more authoritative,

Table 2. The four SSR primer pairs used in the analysis of 75 strawberry accessions, the number of peaks scored, the range of observed peak sizes, and the number of unique genotypes detected

SSR name	Forward primer sequence	Reverse primer sequence	No. of peaks scored	Range of observed peak sizes (bp)	No. of unique genotypes detected
FxaHGA02P13	CCAGGCGCTTGGTCTTGTACTACT	CCCATTTCCCCCAAATCTAACAAT	25	244–302	74
FxaAGA21F11	CAATTCACAATGGCTGATGACGAT	GCACTCAGACATATTTTGGGAGGG	20	134–185	61
EMFv104	TGGAAACATTCTTACATAGCCAAA	CAGACGAGTCCTTCATGTGC	34	98–166	73
EFMvi136	GAGCCTGCTACGCTTTTCTATG	CCTCTGATTCGATGATTTGCT	29 (27)[a]	140–197	65

[a] Two peaks at EMFvi136 were not detected when PCR amplification was performed by the original method of Schuelke (2000). All other genotypes were the same with both methods.

analysis with even a single SSR marker can provide a fair amount of information.

Although peak sizes increased corresponding to the lengths of the sequences we added to the primers, the genotypes at EMFv104 and EMFvi136 obtained by the modified method almost corresponded to those obtained in the previous study (Govan *et al.* 2008) for cultivars analyzed in common. However, some discrepancies such as appearance or disappearance of peaks and slight peak size changes were observed, as were also seen in Brunings *et al.* (2010). Technical variations between studies were considered to be one of the major reasons of such discrepancies. Meanwhile, highly reproducible patterns were observed among experimental methods used in a previous study (Govan *et al.* 2008). Also in this study, reproducible amplification patterns were observed, including between multiple tissue types of the same accession (Fig. 1), and between two PCR methods for three markers as mentioned above. Therefore, although it is required to pay careful attention to the reproducibility of genotype, especially in the comparison of genotypes obtained by different methods or laboratories, useful and convenient genotyping can be performed by choice of appropriate experimental method and SSR markers. For data harmonization between methods or laboratories (Doveri *et al.* 2008, Govan *et al.* 2008, This *et al.* 2004), genotypes of several standard cultivars, and several frequently observed peaks (e.g., 244 in FxaHGA02P13, 146 and 150 in FxaAGA21F11, 102 in EMFv104 and 149 in EMFvi136) will serve as landmarks.

Further, we tested the ability of these four markers to discriminate among 48 full-sib individuals produced from a cross between 'Sagahonoka' and 'Summer-berry', to estimate the resolving power of these markers for highly related individuals. In the set of 48 full-sibs, the number of unique genotypes detected by each marker ranged from 4 (EMFvi136) to 35 (EMFv104; Table 3). These values had the same relative ranking as the number of peaks that segregated among the full-sib progeny: which ranged from 3 (EMFvi136) to 9 (EMFv104). When two markers were combined, the discrimination power increased greatly: by combining any two of the three markers FxaHGA02P13, FxaAGA21F11 and EMFv104, all 48 full-sib individuals could be distinguished. Thus, although it is necessary to pay attention to the degree of differentiation among parents, the combination of just two markers enabled us to discriminate between even highly related individuals.

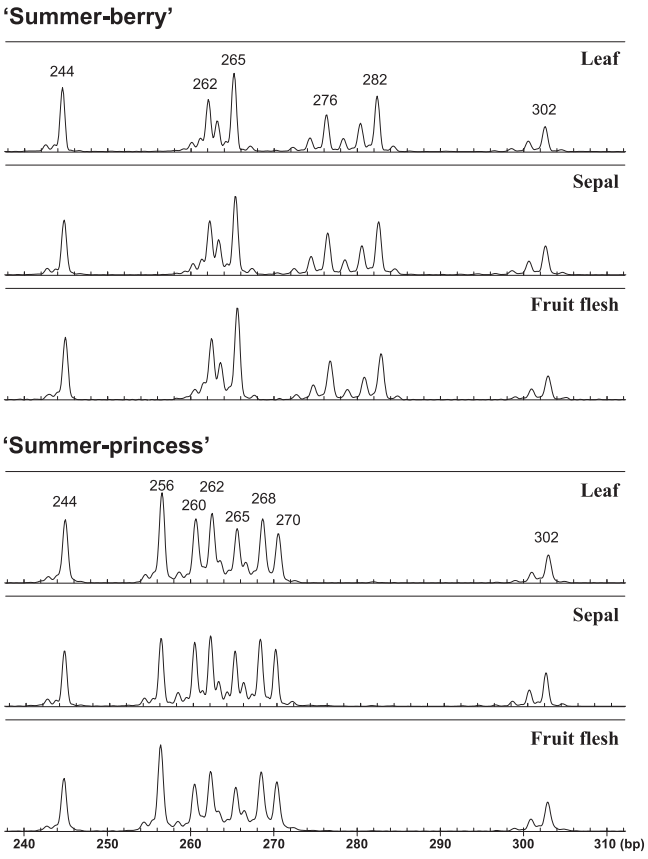

Fig. 1. Amplified fragment patterns of SSR FxaHGA02P13 from different tissue samples of two cultivars. The number above each peak indicates the fragment size.

In addition to cultivar identification, hypervariable SSR markers may be also useful for the elucidation of the genome composition of the octoploid strawberry. Two main hypotheses for the genome composition of *F.* × *ananassa* have been proposed: (1) AAA′A′BBB′B′ (Bringhurst 1990, Kunihisa *et al.* 2005) and (2) AAA′A′BBBB (Senanayake and Bringhurst, 1967, Lerceteau-Köhler *et al.* 2003). Highly polymorphic SSR markers, which are theoretically possible to amplify eight products from four homoeologous loci, may

Table 3. The number of unique genotypes detected in the analysis of 48 full-sibs by single or pairs of SSR markers used in this study

	FxaHGA02P13	FxaAGA21F11	EMFv104	EMFvi136
FxaHGA02P13	18[a]	48	48	43
FxaAGA21F11		28[a]	48	45
EMFv104			35[a]	47
EMFvi136				4[a]

[a] Value for the single SSR marker.

afford direct evidence for mode of inheritance in the cultivated strawberry. For example, in the genotyping of offspring of a cross (A_1A_2 A_3A_4 A_5A_6 $A_7A_8 \times Z_1Z_1$ Z_1Z_1 Z_1Z_1 Z_1Z_1), either one of the two peaks of a parent (e.g., A_1 and A_2 and so on) certainly inherited in the offspring but did not appear simultaneously in an offspring, these two peaks were inferred to be alleles in a pair. If such four pairs ($A_1A_2/A_3A_4/A_5A_6/A_7A_8$) are obtained, it could be one of the direct evidence for the hypothesis AAA′A′BBB′B′. In this study, two peaks of EMFv104 were considered to be alleles in a pair from the analysis of above-mentioned 48 full-sib individuals, but unfortunately, other pairs were not identified due to the overlap of the same size peaks between parents. By selecting mating parents whose peaks are not overlapped, alleles in a pair may be more clearly identified.

PCO analysis of the 75 accessions detected several groups, which reflect taxon, country of origin, and breeding site in Japan, in which limited breeding materials have been repeatedly used to achieve flowering habit suited to the region (Fig. 2). Japanese early-flowering June-bearers were plotted toward the positive end of the PCO1 axis, which accounted for 11.6% of the total variation. Along the PCO2 axis, which accounted for 7.3% of the variation, groups corresponding to wild *Fragaria* species accessions, cultivars bred in America or Europe, and Japanese cultivars could be identified. In Japan, strawberry breeding programs have been conducted to produce cultivars adapted to each region or cropping type. In the warmer regions of Japan, mainly early-flowering June-bearers have been selected because they are adapted to forcing culture, in which fruits are usually harvested beginning in the early winter. On the other hand, in the cooler regions of Japan, mainly late-flowering June-bearers have been selected because they are better adapted to semi-forcing or open culture, in which fruits are harvested in spring and early-summer. In cooler regions, the breeding and use of everbearing cultivars is also popular. As a result of the repeated use of breeding materials suited to particular breeding objectives, differences in allelic composition between the early-flowering June-bearers and the late-flowering June-bearers and everbearers may have occurred. Several everbearers and late-flowering June-bearers that were derived from early-flowering June-bearers, such as ‘Summer-princess’ (accession 63; offspring of early-flowering ‘Nyoho’), ‘Raiho’ (accession 53; offspring of a seedling of ‘Nyoho’) and ‘Oze-akarin’ (accession 48; a grandchild of early-flowering ‘Tochiotome’), were plotted in the proximity of early-flowering June-bearers.

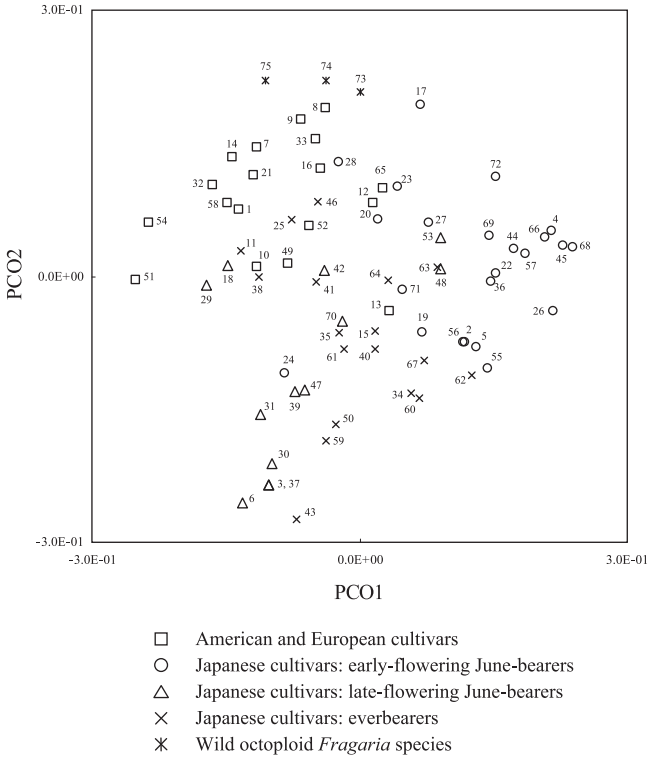

Fig. 2. Principal-coordinate (PCO) analysis of 75 accessions consisting of 72 *F.* × *ananassa* cultivars or lines and three octoploid *Fragaria* species accessions analyzed by four SSR markers. The first (PCO1) and second (PCO2) axes explain 11.6% and 7.3% of the total variation, respectively. Numbers correspond to accession numbers in Table 1.

Three accessions of wild octoploid species *F. chiloensis* and *F. virginiana* (accessions 73, 74 and 75) were plotted at the edge of the group in Fig. 2. Although *F.* × *ananassa* is an interspecific hybrid of *F. chiloensis* and *F. virginiana*, only a few genotypes of these species contributed to the establishment of *F.* × *ananassa* (Dale and Sjulin 1990). Probably for this reason, the *F. chiloensis* and *F. virginiana* accessions used in this study showed a different allelic composition from the *F.* × *ananassa* accessions. Old cultivars such as the first Japanese cultivar, ‘Fukuba’ (accession 17; released in 1899), ‘Marshall’ (accession 33, released in 1893), which was the oldest among cultivars analyzed in this study, ‘Cambridge Favourite’ (accession 9; released in 1947) and ‘Blakemore’ (accession 7; released in 1929), were plotted closer to wild *Fragaria* accessions than almost all of the other more recent cultivars. Although further investigation with more markers is needed, such pattern may reflect the historical transition of genetic composition in strawberry cultivars. Also, the fact that most of the early-flowering June-bearing cultivars in Japan after ‘Toyonoka’(accession 69; registered in 1984) and ‘Nyoho’ (accession 45; registered in 1985) converged in the right side of Fig. 2, while older early-flowering June-bearers such as ‘Fukuba’, ‘Hogyoku’ (accession 23), ‘Hatsukuni’ (accession 20), ‘Uzushio’ (accession

71), 'Haruyoi' (accession 19), 'Hokowase' (accession 24) and 'Reiko' (accession 55) were scattered around the position, may suggest the genetic homogenization in recent years.

This study identified highly polymorphic SSR markers which can be used for the identification of Japanese strawberry cultivars. Together with other available markers, these SSRs will contribute to the management of genetic resources of strawberries and the protection of breeders' rights.

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