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Clone identification in Japanese flowering cherry (*Prunus* subgenus *Cerasus*) cultivars using nuclear SSR markers

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Numerous cultivars of Japanese flowering cherry (*Prunus* subgenus *Cerasus*) are recognized, but in many cases they are difficult to distinguish morphologically. Therefore, we evaluated the clonal status of 215 designated cultivars using 17 SSR markers. More than half the cultivars were morphologically distinct and had unique genotypes. However, 22 cultivars were found to consist of multiple clones, which probably originate from the chance seedlings, suggesting that their unique characteristics have not been maintained through propagation by grafting alone. We also identified 23 groups consisting of two or more cultivars with identical genotypes. Most members of these groups were putatively synonymously related and morphologically identical. However, some of them were probably derived from bud sport mutants and had distinct morphologies. SSR marker analysis provided useful insights into the clonal status of the examined Japanese flowering cherry cultivars and proved to be a useful tool for cultivar characterization.

Key Words: *Cerasus*, clone identification, cultivars, *Prunus*, SSR, microsatellite, taxonomy.

Introduction

Flowering cherries (members of *Prunus* subgenus *Cerasus*, Rosaceae) are the most popular ornamental trees in Japan, and have been cultivated for more than 1,000 years (Flower Association of Japan 1982, Kuitert 1999). There are more than 200 traditional cultivars in Japan (Kobayashi 1992), which mainly differ in flower color, form, size and number of petals. Some have been exported widely and are grown world-wide. Most cultivars have been clonally propagated to avoid the dilution or loss of their unique characteristics and many are believed to have originated from native Japanese taxa (Kawasaki 1993, Koidzumi 1913, Kuitert 1999, Miyoshi 1916). Some are considered to be related with *P. campanulata*, which is endemic to Taiwan, southern China and neighboring countries and *P. pseudo-cerasus*, a native of China (Kawasaki 1993). The first morphological studies on Japanese flowering cherry cultivars were conducted in the early 20th century, focusing on the cultivars in Arakawa-zutsumi (Koidzumi 1913, Miyoshi 1916, Wilson 1916). Later works established a taxonomy for the cultivars (Flower Association of Japan 1982, Kawasaki 1993) that is now widely accepted (Ohba *et al.* 2007). However, it is difficult to classify the cultivars' morphological characteristics, which can vary with weather and nutrient conditions and are sometimes affected by their rootstocks. Consequently, seemingly different cultivars may really be different clones of the same cultivar, i.e. synonymous cultivars (Ohba *et al.* 2007). Such incorrect classification may also arise from management errors such as mislabeling and/or mistransplanting. Consequently, morphology-based classification of cultivars is complicated and needs to be reviewed thoroughly. While many taxonomists are primarily interested in the origin and pedigree of the cultivars, which are reflected in their morphological traits and were the primary focus of previous studies, the key objective in contemporary research is to determine the trees' clonal status.

Molecular genetics provides powerful tools for identifying clones in plant cultivars. In particular, nuclear simple sequence repeat (SSR) markers have proven to be extremely useful for characterizing cultivars. The key advantages of SSR markers are their codominant mode of inheritance and

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hypervariability, which make them ideal for diverse applications (Goldstein and Schlötterer 1999). They have therefore found widespread use for identifying clones within cultivars of many fruit tree species that are closely related to the Japanese flowering cherry. These include sweet cherry, *Prunus avium* (Wünsch and Hormaza 2002); peach, *Prunus persica* (Aranzana *et al.* 2003, Yamamoto *et al.* 2003) and the apricot species, *Prunus armeniaca* and *Prunus mume* (Maghuly *et al.* 2005 and Hayashi *et al.* 2008, respectively). Molecular genetics techniques have not been applied extensively to Japanese flowering cherry cultivars. Hattori *et al.* (2002) analyzed several cultivars and identified two pairs of putative synonymous cultivars, each of which has identical genotypes, and the clonal status of *P.* ×*yedoensis* 'Yedoensis' (Somei-yoshino), the most common cultivar in Japan, has been confirmed by molecular genetic approaches (Iketani *et al.* 2007, Innan *et al.* 1995). However, the clonal status of many other cultivars remains unclear (Ohba *et al.* 2007).

The objective of the study reported here was to investigate the clonal status of selected Japanese flowering cherry cultivars using SSR markers. We increased the reliability of the gathered data by performing comparative analyses of cultivars growing at multiple locations. Trees grown at the sampling sites were separated into distinct cultivated lines, for each of which archived records detailing its supplier and nominal designation were available. We therefore began by checking the accuracy of the records for each cultivated line, then genotyped the lines and compared the genotypic and nominal classifications.

Materials and Methods

Sample collection and archival records

Samples were obtained from the collections of cultivated taxa maintained in the Tama Forest Science Garden (TFSG; Hachioji, Tokyo, Japan) of the Forestry and Forest Products Research Institute (FFPRI), National Institute of Genetics (NIG; Mishima, Shizuoka, Japan) and Shinjuku Gyoen National Garden (SGNG; Shinjuku, Tokyo, Japan). A total of 1986 trees were sampled—1567 from the TFSG, 331 from the NIG and 88 from the SGNG. The morphological traits of the sampled trees growing in the field were recorded and sampled leaves were frozen immediately then stored frozen until DNA extraction. We also prepared the flower specimens of almost sampled trees and deposited in the herbarium of TFSG (herbarium code: TFA).

An archival record of each cultivated tree is extremely important information for identification of the cultivar. Therefore, we identified the nominal designations and supplier's names from the internal document or published papers (Ministry of Agriculture, Forestry and Fisheries 1981, Moriwaki and Katsuki 2011). The suppliers' names are listed in Supplemental Table 1. Some of the trees growing at TFSG that were sampled had been imported from NIG or SGNG; in these cases, the supplier's name was recorded as NIG or SGNS, as appropriate. The nominal designation for each tree was the cultivar name recorded in the archives; many of these cultivar names were published in one of 10 scientific papers (Asari 1995, Hayashi 1985, Kawasaki 1959, 1993, Kimura 1982, Kubota 1982, Ministry of Agriculture, Forestry and Fisheries 1981, Ohwi 1973, Tamura and Iyama 1989 and a chapter written by Tanaka, H. in Ohba 2007). We observed flower characteristics especially calyx tube and calyx lobe according to the description of Kawasaki (1993). When the nominal designation and observed characteristics of sampled tree matched with the names and morphological characteristics described in these papers, we identified it as a cultivar. However, some trees could not be confirmed only from their morphological characteristics. The notation of the scientific name in this paper followed that of Kawasaki (1993), but the cultivar epithets of Kawasaki (1993) were described using "cv.". The usage of "cv." is not permitted in the current international code of nomenclature for cultivated plants (Brickell *et al.* 2009). Therefore, cultivar epithets were corrected by enclosing them within single quotation marks. The scientific name that should be revised elsewhere exists, but is regarded as a future issue.

DNA analysis

DNA was isolated from leaves of each sampled tree following Murray and Thompson (1980). Genotypes of the samples were scored using three genomic SSRs (Ishio *et al.* patent pending) and 14 SSRs in expressed sequence tags (ESTs), i.e. EST-SSRs (Tabe 1). Eleven of the EST-SSRs were previously reported by Tsuda *et al.* (2009) and one by Xu *et al.* (2004). The other two were developed in the course of this study, according to the procedure of Tsuda *et al.* (2009); their designations and the corresponding primers are DN556408 (forward primer, 5′-attcttcttccgccacttctgaat-3′; reverse primer, 5′-gtttagatcacagcacgcgaaaatg-3′) and DW358868 (forward primer, 5′-attgatttcggacccataaaaccc-3′; reverse primer, 5′-gtttacaacatcacgtacgggcctc-3′). PCR amplification was performed in 2 μl reaction mixtures containing 5 ng of template DNA, 1× Qiagen multiplex PCR master mix and the appropriate primers. Multiplex PCR was performed using conditions recommended by Qiagen. PCR products were separated using an ABI 3100 Avant Genetic Analyzer with the GeneScan software system or an ABI 3130 Genetic Analyzer (Applied biosystems, USA). To avoid the electrophoresis results possibly being influenced by the type of sequencer used, we employed the same sequencer for all analyses of amplification products generated using the same primer combination. The genotypes were scored using Genotyper or GeneMapper (Applied biosystems, USA).

Genotypic classification

The tree samples were genotyped using the 17 SSRs described above. Due to their historical importance in the analysis of Japanese flowering cherry cultivars, this paper

Table 1. Variability parameters calculated for 17 SSR markers in the Japanese flowering cherry cultivars: number of alleles (A), expected heterozygosity (H_E) and power of discrimination (PD)

Locus	All cultivars (222 clones)			*Prunus lannesiana* (76 clones)			*Prunus jamasakura* (20 clones)			*Prunus pendula* (21 clones)			*Prunus ×yedoensis* (27 clones)			Reference[a]
	A	H_E	PD	A	H_E	PD	A	H_E	PD	A	H_E	PD	A	H_E	PD	
AM287648	11	0.74	0.89	7	0.65	0.82	7	0.74	0.88	3	0.26	0.45	4	0.29	0.38	1
AM287842	14	0.78	0.92	9	0.53	0.79	10	0.83	0.91	4	0.69	0.83	5	0.55	0.73	1
AM288205	12	0.71	0.87	3	0.57	0.71	4	0.49	0.71	2	0.05	0.09	5	0.54	0.71	1
AM290339	5	0.12	0.20	2	0.02	0.05	3	0.14	0.27	–[b]	–	–	1	–	–	1
DN553427	6	0.28	0.47	4	0.25	0.45	4	0.27	0.47	2	0.14	0.24	3	0.28	0.47	1
DN554499	7	0.42	0.59	4	0.22	0.35	4	0.52	0.70	1	–	–	3	0.47	0.53	1
DN556408	5	0.66	0.83	5	0.51	0.61	3	0.51	0.68	2	0.14	0.24	3	0.54	0.57	2
DW358868	8	0.72	0.87	5	0.55	0.75	4	0.69	0.85	1	–	–	4	0.62	0.81	2
DY640364	4	0.14	0.21	2	0.01	0.03	2	0.14	0.26	2	0.05	0.09	1	–	–	1
DY640849	21	0.80	0.93	13	0.71	0.88	8	0.71	0.75	5	0.27	0.40	8	0.61	0.80	1
DY646168	5	0.55	0.71	5	0.43	0.61	4	0.56	0.75	–	–	–	4	0.23	0.33	1
DY647422	4	0.35	0.54	4	0.46	0.62	2	0.47	0.57	2	0.14	0.24	2	0.13	0.25	1
DY652293	5	0.71	0.87	5	0.57	0.72	5	0.67	0.76	3	0.14	0.18	4	0.57	0.67	1
ASSR1	4	0.35	0.50	2	0.01	0.03	2	0.14	0.26	2	0.14	0.24	2	0.40	0.49	3
sk1-1	28	0.82	0.94	11	0.55	0.75	8	0.79	0.89	10	0.86	0.94	7	0.72	0.81	4
sk3-1	38	0.93	0.98	23	0.88	0.97	18	0.89	0.92	8	0.73	0.88	11	0.82	0.87	4
sk4-1	14	0.76	0.88	7	0.60	0.78	5	0.78	0.90	4	0.66	0.75	5	0.65	0.74	4
Mean	11.2	0.58	0.72	6.5	0.40	0.58	5.5	0.55	0.67	3.1	0.33	0.37	4.2	0.50	0.54	
			(2.18×10^{-13})[c]			(2.54×10^{-9})			(7.05×10^{-11})			(2.16×10^{-5})			(1.12×10^{-7})	

[a] 1, Tsuda *et al.* 2009; 2, this study; 3, Xu *et al.* 2004; 4, Ishio *et al.* patent pending.

[b] "–" indicates that the variability parameters could not be calculated because there was little or no detectable amplification or the amplification products were monomorphic.

[c] The probability of confusion, calculated using the PD values, is shown in parentheses.

focuses exclusively on the genotypes of the popularly propagated cultivars described in the 10 scientific papers listed in the section on sample collection (Kawasaki 1993 and others). In the end, a total of 1479 samples were analyzed—1164 from the TFSG, 251 from the NIG and 64 from the SGNG, representing 693 cultivated lines. Because the information of archival records is important, we also included the lines with archival records disaccorded with cultivar name.

Many of the other samples, whose morphological characteristics do not conform with the descriptions of cultivars in any of the 10 papers listed above and thus data are not shown in this paper, were taken from so-called "meiboku (in Japanese)" trees that are not widely propagated, but are nevertheless famous for their very old age, cultural history and/or beautiful morphology. The results obtained from analyses of these sampleswill be discussed elsewhere.

Statistical analyses

The parameters used to evaluate the information obtained by genotyping the SSR loci were the number of alleles (A) and the expected heterozygosity ($H_E = 1 - \Sigma p_i^2$, where p_i is the frequency of the ith allele). Japanese flowering cherry cultivars include triploids (Iwatsubo *et al.* 2002, 2003, 2004). In such cases, the polysat package of R (R Development Core Team 2010) was used to calculate the allele frequencies at each locus, under the assumption that in a partially heterozygous genotype, all alleles have an equal chance of being present in more than one copy. The ability to discriminate between two random clones for each locus was estimated in terms of the power of discrimination ($PD = 1 - \Sigma g_i^2$, where g_i is the frequency of the ith genotype) (Kloosterman *et al.* 1993). The PD values were also used to calculate the probability of confusion [$C = \Pi(1 - PD_i)$, where PD_i is the PD value of the ith locus], i.e. the probability that any two cultivars would have identical SSR genotypes by chance alone, considering all loci. These indices were calculated for every clone identified and for each taxon.

Results

The 17 SSR markers used in this study produced amplified fragments with sizes ranging from 119 to 356 bp (Supplemental Table 2). Most SSR markers yielded one or two amplified fragments, but putative triploid cultivars yielded three fragments for some SSRs. No cultivars yielded more than four fragments. The ploidy levels were also estimated by SSR analysis, i.e., maximum number of alleles per locus (Supplemental Table 3). In all, 217 cases could be checked against the data of somatic chromosome counts reported in Iwatsubo *et al.* (2002, 2003, 2004), and only six of them had inconsistent results. No amplification products were obtained for some loci in some cultivars; notably the AM290339 and DY646168 SSRs yielded no amplification products in *P. pendula*. Each new clone identified was

assigned a designation of the form Cer001 or Cer002 etc. on the basis of their SSR genotype (Supplemental Table 3). Individual samples were identified as belonging to specific clones in a conservative fashion; samples whose genotypes deviated from that for a previously-identified clone at only one locus were assigned branch numbers to indicate this difference from the archetype. Such samples were therefore given designations of the form Cer011-i or Cer011-ii. However, for subsequent analyses such deviating samples were treated as the same clone as the archetype, i.e. a sample designated Cer011-i would be treated as a clone of Cer011 (see discussion). In all, we identified 222 different clones (Supplemental Table 3).

The archived records for each cultivated line were checked against their morphological characteristics and the accuracy of the recorded data was evaluated (Supplemental Table 3). Such evaluations were only possible for lines whose nominal designations were (roughly) consistent with the scientific name of the corresponding cultivar; for cultivars with alternative names (e.g. 'Sendai-shidare', which is of the Cer072 genotype, is also known as "Fugen-shidare", "Yamazakura-shidare" and "Yoshino-shidare"), all of the nominal designations were considered. The archived records for a cultivated line were considered to be correct if the morphological characteristics suggested by its nominal designation were consistent with its appearance; such lines are underlined in Supplemental Table 3. The archived records of lines for which this was not the case were considered to be incorrect; such lines are marked with the "#" symbol in Supplemental Table 3. The nominal designations recorded in the archives were correct for 1101 trees representing 494 cultivated lines, but were incorrect for 211 trees representing 118 cultivated lines. It was not possible to determine the accuracy of the nominal designations for cultivated lines that have not been assigned a scientific name, i.e. that have not been described taxonomically. In Supplemental Table 3, lines of this sort are indicated by an asterisk; one such line is "Hirano-nadeshiko", which was assigned to the clone Cer014. Similarly, it was not possible to evaluate the accuracy of designations for cultivated lines whose nominal designations had been lost; such lines carry the legend "No Name" in Supplemental Table 3. Overall, there were 167 trees (representing 81 cultivated lines) for which it was not possible to assess the accuracy of the archival record.

The scientific names for the cultivars were assigned on the basis of their morphological characteristics, mainly using information provided by the 10 papers (i.e. Kawasaki 1993 and others) cited above and the archival records, then assigned to respective taxa on the basis of the morphological criteria (Supplemental Table 3). The 222 clones identified among our samples were classified as representing 215 cultivars on the basis of their morphological characteristics and archival records. *Prunus lannesiana* was represented by 76 clones (Cer001 to Cer076), *P. lannesiana* var. *speciosa* by 11 clones (Cer077 to Cer087), *P. jamasakura* by 20 clones (Cer088 to Cer107), *P. sargentii* by one clone (Cer108), *P. verecunda* by five clones (Cer109 to Cer113), *P.* ×*sieboldii* by one clone (Cer114), *P. apetla* var. *pilosa* by one clone (Cer115), *P. incisa* var. *incisa* by nine clones (Cer116 to Cer124), *P. incisa* var. *kinkiensis* by two clones (Cer125 and Cer126), *P.* ×*parvifolia* by four clones (Cer127 to Cer130), *P.* ×*furuseana* by one clone (Cer131), *P.* ×*syodoi* by two clones (Cer132 and Cer133), *P.* ×*tajimensis* by one clone (Cer134), *P.* ×*subhirtella* by 10 clones (Cer135 to Cer144), *P. pendula* by 21 clones (Cer145 to Cer165), *P.* ×*sacra* by two clones (Cer166 and Cer167), *P.* ×*yedoensis* by 27 clones (Cer168 to Cer194), *Prunus* (hybrids between two or more species) by eight clones (Cer195 to Cer202), *P. campanulata* by one clone (Cer203), *P.* ×*kanzakura* by nine clones (Cer204 to Cer212), *P.* ×*introrsa* by seven clones (Cer213 to Cer219), *P.* ×*miyoshii* by one clone (Cer220) and *P.* ×*takenakae* by two clones (Cer221 and Cer222). On the basis of the sampled trees' genotypes, morphology and archived data, three distinct types of clonal status were identified (Supplemental Table 3). The results are summarized in Table 2. Of the cultivars examined, 140 were of Type A, differing in both genotypically and morphologically, thus each of these 140 cultivars consisted of a single clone. Twenty-two of the examined cultivars consisted of multiple clones, which have similar or near-identical morphologies but exhibit some genotype variation (Type B). In addition, there were 23 groups of two or more distinct cultivars with identical genotypes (Type C). In most cases, the members of a given group of Type C cultivars had identical morphologies, but some were clearly distinct. Six cultivars, which are marked with an asterisk in Table 2, belonged to both Types B and C.

The variability parameters are shown in Table 1. Samples whose genotypes differed only at a single locus were treated as being from identical clones. Consequently, the genotype data for these samples were discarded only when calculating the variability parameters for the locus. The number of alleles ranged from four to 38 per locus, giving a total of 191 alleles at the 17 SSR loci. The expected heterozygosity ranged from 0.12 to 0.93, with a mean value of 0.58. Using the PD values, we calculated the probability of confusion to be 2.18×10^{-13}. The indices were also calculated for all taxa consisting of more than 20 clones, which are considered to be an adequate sample size. The taxa that satisfied this criterion are *P. lannesiana*, *P. jamasakura*, *P. pendula* and *P.* ×*yedoensis*; their variability parameters are also shown in Table 1.

Discussion

The SSR markers used in the study showed a high degree of polymorphism in the Japanese flowering cherry cultivars, which have been produced by crossbreeding wild taxa (Kawasaki 1993). The probability of confusion calculated from the PD values was 2.18×10^{-13}; much lower than that determined for peach (*P. persica*) cultivars (2.32×10^{-9}) by Aranzana *et al.* (2003) in an analysis of similar numbers of

Table 2. The clonal status of the studied Japanese flowering cherry cultivars. Type A cultivars differ both genotypically and morphologically, i.e., each cultivar consists of a single clone. Type B cultivars consist of multiple clones that exhibit similar or near-identical morphologies but differ genetically. Type C cultivars have genotypes that are identical to at least one other cultivar; more details are provided in Supplemental Table 3.

Groups	Name[a]
Type A	
Prunus lannesiana	'Arasiyama', 'Formosissima' (Benitorano-o), 'Gosiozakura', 'Hisakura' (Ichiyo), 'Kirin', 'Kokeshimidsu', 'Koshioyama', 'Mikurumakaisi', 'Rubida' (Bendono), 'Sekiyama' (Kanzan), 'Shujaku' (Suzaku), 'Similis' (Taguiarashi), 'Sobanzakura' (Itsukayama), 'Spiralis' (Uzu-zakura), 'Superba' (Shogetsu), 'Nigrescens' (Usuzumi), 'Moutan' (Botan), 'Gosho-mikurumagaeshi', 'Hirano-nezame', 'Imose', 'Kodaiji', 'Taoyame', 'Yadakemurasaki', 'Aratama', 'Beni-yutaka', 'Hanagasa', 'Matsumae-benihigoromo', 'Matsumae-benitamae', 'Matsumae-hanazomei', 'Matsumae-hayazaki', 'Matsumae-sarasa', 'Shizuka', 'Chihara-zakura', 'Fudanzakura', 'Gosho-odora' (Gosho-nioi), 'Goten-nioi', 'Iyo-kumagai', 'Mirabilis' (Omura-zakura), 'Mitsukabizakura', 'Multipetala' (Najima-zakura), 'Ohta-zakura', 'Oshusatozakura', 'Polycarpa' (Fuku-zakura), 'Sendai-shidare', 'Sphaerantha' (Kenrokuen-kikuzakura), 'Tokyo' (Tokyo-zakura), 'Vexillifera' (Hakusan-hata-zakura), 'Yaenioi'
Prunus lannesiana var. *speciosa*	'Akami-ohshima', 'Formosa' (Shiokaze-zakura), 'Glauca' (Minakami), 'Izuzakura', 'Kanzaki-ohshima', 'Plena' (Yae-oshima-zakura), 'Shin-sumizome'
Prunus jamasakura	'Agishi-kogikuzakura', 'Beninanden', 'Fomasa' (Yaesakon-zakura), 'Gijozakura' (Gijyoji-gijozakura), 'Haguiensis' (Ketanoshiro-kikuzakura), 'Heteroflora' (Nido-zakura), 'Hiuchidani-kikuzakura', 'Humilis' (Wakakino-sakura), 'Ichihara' (Ichiharatorano-o), 'Imperialis' (Dairino-sakura), 'Iyo-usuzumi', 'Kongo' (Kongo-zakura), 'Kotohira', 'Nahohiana' (Konohana-zakura), 'Octopes' (Yatsubusa-zakura), 'Raikouji-kikuzakura', 'Sanozakura', 'Sendaiya', 'Sunpu-zakura'
Prunus sargentii	'Kushiroyae' (Kushiroyae)
Prunus verecunda	'Kanzashi-zakura', 'Norioi' (Kataoka-zakura), 'Pendula' (Kirifuri-zakura)
Prunus ×*sieboldii*	'Caespitosa' (Takasago)
Prunus apetala var. *pilosa*	'Multipetala' (Hinagiku-zakura)
Prunus incisa var. *incisa*	'Bellura' (Asagiri-zakura), 'Fujikikuzakura', 'Longipes' (Nagarano-mamezakura), 'Oshidori' (Oshidori-zakura), 'Plena' (Yaeno-mamezakura), 'Yamadei' (Midori-zakura)
Prunus ×*parvifolia*	'Globosa' (Amedama-zakura), 'Pendula' (Shidare-kobazakura), 'Parvifolia' (Fuyuzakura), 'Umineko'
Prunus ×*furuseana*	'Incisoides' (Bonbori-zakura)
Prunus ×*syodoi*	'Ganman-zakura', 'Shodoi' (Shodo-zakura)
Prunus ×*tajimensis*	'Tajimensis' (Yumura)
Prunus ×*subhirtella*	'Omoigawa'
Prunus pendula	'Plena-rosea' (Yaebeni-shidare), 'Ujou-shidare'
Prunus ×*sacra*	'Mochidzukiana' (Mochizuki-zakura), 'Sacra' (Katte-zakura)
Prunus ×*yedoensis*	'Amagi-yoshino', 'America', 'Candida' (Usuge-oshima), 'Funabara-yoshino', 'Hayazaki-oshima', 'Izu-yoshino', Kichijouji, 'Kurama-zakura', 'Mikado-yoshino', 'Mishima-zakura', 'Morioka-pendula' (Morioka-shidare), 'Naniwa-zakura', 'Pendula' (Shidare-okusaizakura), 'Perpendens' (Shidare-someiyoshino), 'Sakabai' (Sendai-yoshino), 'Sakuyahime', 'Sasabe-zakura', 'Shouwa-zakura', 'Somei-higan', 'Somei-nioi', 'Sotorihime', 'Suruga-zakura', 'Syuzenzi-zakura', 'Waseyoshino'
Prunus (hybrids between two or more species)	'Kobuku-zakura', 'Kursar, 'Manadzuru-littorea' (Mizutama-zakura), 'Moniwana' (Moniwa-zakura), 'Okame', 'Rubriflora' (Benizuru-zakura), 'Takinoensis' (Takino-zakura), 'Youkou'
Prunus campanulata	'Ryukyu-hizakura' (Ryukyu-kanhizakura)
Prunus ×*kanzakura*	'Oh-kanzakura', 'Rubescens' (Shuzenji-kanzakura), 'Yokohama-hizakura'
Prunus ×*introrsa*	'Hina-zakura', 'Tagabeni, 'Yayoi-zakura'
Prunus ×*miyoshii*	'Ambigua' (Taizanfukun)
Prunus ×*takenakae*	'Takenakae' (Tokai-zakura)
Type B	
Prunus lannesiana	'Arakawaensis' (Arakawanioi), 'Kenrokuen-kumagai'*, 'Versicolor' (Yae-akebono)*, 'Azuma-nishiki'*, 'Fasciculata' (Itokukuri)*, 'Shibayama', 'Beni-tamanishiki'
Prunus lannesiana var. *speciosa*	'Semiplena' (Usugasane-oshima), 'Yaebeni-ohshima'
Prunus verecunda	'Antiqua' (Narano-yaezakura)
Prunus incisa var. *incisa*	'Urceolata' (Obana-mamezakura)
Prunus incisa var. *kinkiensis*	'Viridicalyx' (Midori-kinkimamezakura)
Prunus ×*subhirtella*	'Autumnalis' (Jugatsu-zakura)*, 'Subhirtella' (Kohigan)*, 'Semperflorens' (Shikizakura), 'Hisauchiana' (Yabu-zakura),
Prunus pendula	'Pendula' (Shidare-zakura)

Table 2. (continued)

Groups	Name[a]
Prunus ×*yedoensis*	'Pilosa' (Ke-oshima-zakura)
Prunus ×*kanzakura*	'Kawazu-zakura', 'Kanzakura'
Prunus ×*introrsa*	'Introrsa' (Tsubaki-kanzakura), 'Myoshoji'
Type C	
Prunus lannesiana	'Affinis' (Jyonioi)/'Hotate', 'Albo-rosea' (Fugenzo)/'Kusimana' (Kushima-zakura), 'Cataracta' (Takinioi)/'Gozanomanioi'/'Sancta' (Meigetsu)/'Surugadai-odora' (Surugadainioi), 'Chosiuhizakura'/'Kenrokuen-kumagai'*, 'Contorta' (Fukurokuju)/'Versicolor' (Yae-akebono)*, 'Versicolor' (Yae-akebono)*/'Erecta' (Amanogawa), 'Eigenji'/'Fusa-zakura'/'Gioiko'/'Grandiflora' (Ukon), 'Kongosan'/'Purpurea' (Murasaki-zakura), 'Masuyama'/'Shiogama' (Shiogama-zakura)/'Suisho', 'Azuma-nishiki'*/'Fasciculata' (Itokukuri)*/'Hakusan-ohtemari'/'Nobilis' (Edo)/'Yae-benitorano-o', 'Azuma-nishiki'*/'Fasciculata' (Itokukuri)*/'Mollis' (Yokihi), 'Kariginu'/'Okinazakura'/'Sirayuki', 'Amayadori'/'Sirotae', 'Daimin'/'Wasinowo', 'Komatunagi'/'Kurumadome'/'Taihaku', 'Multiplex' (Mazakura)/'Candida' (Ariake)/'Ojochin'/'Senriko', 'Juzukakezakura' (Baigoji-juzukake-zakura)/'Tsukubane', 'Kibune-uzu'/'Omuro-ariake', 'Kinashi-chigozakura'/'Nison-in' (Nison-in-fugenzo), 'Ohsawazakura'/'Shonaden'
Prunus jamasakura	'Goshinzakura'/'Hiyoshizakura'
Prunus ×*subhirtella*	'Autumnalis' (Jugatsu-zakura)*/'Higan-dai-zakura'/'Subhirtella' (Kohigan)*/'Shofukuji' (Shofukuji-zakura)/'Yaebeni-higan' (Yaebeni-higan)
Prunus ×*yedoensis*	'Yedoensis' (Somei-yoshino)/'Somei-beni'

[a] C-Type cultivars with identical genotypes are separated by slashes. Cultivars marked with an asterisk belong to both Types B and C.

cultivars and markers. The mean expected heterozygosity value for the SSR markers used was 0.58, similar to reported values for fruit cultivars of other *Prunus* species; 0.49 (Wünsch and Hormaza 2002), 0.5 (Aranzana *et al.* 2003), 0.74 (Maghuly *et al.* 2005) and 0.68 (Hayashi *et al.* 2008). Among taxa, the genetic diversities of *P. pendula* and *P.* ×*yedoensis* (in both of which monomorphic markers were detected) were lower than those of *P. lannesiana* and *P. jamasakura* (Table 1). Some cultivars of *P.* ×*yedoensis* have been shown to be offspring of 'Yedoensis' (Iketani *et al.* 2007). Thus, the narrow genetic base could be ascribed to the use of a limited number of family lineages as parents. Some others have been artificially produced using both *P. pendula* and *P. lannesiana* var. *speciosa* (Tamura and Iyama 1989). Thus, the lower genetic diversities observed in the cultivated trees of *P. pendula* and *P.* ×*yedoensis* could be attributed to the fact that the wild populations of *P. pendula*, which may have been used in the breeding process, may have lower genetic diversity than other wild *Prunus* species. Furthermore, no amplification products were observed at two SSR markers in *P. pendula* and these cultivars may also carry null alleles at other marker loci. A previous molecular phylogenetic study of Japanese wild flowering cherries revealed that *P. pendula* is distantly related to various wild Japanese taxa (Ohta *et al.* 2007), which might explain the low applicability of the SSR markers used. Given these considerations, the genotype data for cultivars related to *P. pendula* should be treated with caution. The ploidy levels estimated from our data were consistent with those of Iwatsubo *et al.* (2002, 2003, 2004), although some cultivars were not (Supplemental Table 3). The four cases, in which the ploidy levels are underestimated in our study, can be tested by genotyping using a larger number of SSR markers and/or more polymorphic markers. The other two cases could be due to amplification of the duplicated SSR regions.

The genotyping of the selected SSR loci revealed three types of clonal status among the Japanese flowering cherry cultivars (Table 2). More than half of the studied cultivars were of Type A, consisting of a single clone, suggesting that they have been propagated exclusively by grafting and that this is responsible for the maintenance of their unique characteristics. In addition, several Type B cultivars, consisting of multiple clones, were identified. While these clonal differences were difficult to identify on the basis of morphological variation alone, they were readily apparent from the SSR analysis. This genotypic variation probably originates from chance seedlings, suggesting that the characteristics of these cultivars have not been maintained through propagation by grafting alone, although this would depend to some extent on how the cultivars are defined. Finally, we identified groups of two or more cultivars with identical genotypes (Type C in Table 2). Some of the C-Type cultivars were also inevitably assigned to Type B. This is the best observation at present because it depends on how the cultivars are defined. In many cases of the C-Type cultivars, the members of each group had been previously suggested to be synonymous cultivars because they do not differ morphologically, but they have been regarded as different cultivars for a long time (Ohba *et al.* 2007). Our results strongly suggest that these groups are synonymous. However, some Type C groups consisted of cultivars that are morphologically distinct but have identical genotypes, suggesting that they are derived from bud sport mutants or that the SSR markers used provide insufficient resolution to discriminate between them. The 'Grandiflora' (Ukon) and 'Gioiko' cultivars of *P. lannesiana* are C-Type cultivars with yellow or yellowish green flowers (Cer011-ii) and have often been suggested to derive from bud sport mutants due to their morphological

traits (Katsuki 2001, Moriwaki and Katsuki 2011). It is possible that they are bud sport mutants of the 'Fusa-zakura' and 'Eigenji' cultivars of *P. lannesiana*, which are putative synonymous cultivars with white double flowers, because they differ only at one of the examined loci (Cer011-i). The 'Somei-beni' cultivar of *P.* ×*yedoensis* is a bud sport mutant of the original cultivar 'Yedoensis' (Somei-yoshino), which is of the Cer194 genotype (Tamura and Iyama 1989). Our results strongly suggest that these two pairs are derived from bud sport mutants rather than being artifacts arising from the low resolution of our SSR analysis. Two pairs of cultivars of *P. lannesiana*, one pair 'Multiplex' (Ma-zakura) and three putative synonymous cultivars, 'Candida' (Ariake), 'Ojochin' and 'Senriko' (Cer039-i and -ii) and the other pair 'Omuro-ariake' and 'Kibune-uzu' (Cer044), might be also derived from bud sport mutants, given their distinguishable morphologies. The 'Shofukuji' (Shofukuji-zakura), 'Higandai-zakura', 'Autumnalis' (Jugatsu-zakura), 'Subhirtella' (Kohigan) and 'Yaebeni-higan' cultivars of *Prunus* ×*subhirtella* also have near-identical genotypes (Cer138-i to -iv) but are morphologically distinct. In this case, it seems that the SSR markers used were insufficient to discriminate them because they are related to *P. pendula* (Ohba *et al.* 2007), in which the marker polymorphism is very low (Table 1). Some other cultivars might also be derived from bud sport mutants; a possibility that could be tested by more detailed observation of the cultivars' morphological characteristics and genotyping using a larger number of SSR markers.

A possibility that should be considered is that the genotype mismatches at a single locus may have originated from clonal differences, such as those arising from chance seedlings, rather than (as assumed) incidental mutations. However, most of the clones that exhibited only a single genotypic mismatch did not differ morphologically; morphological differences were much more common between clones with mismatches at multiple loci. Thus, this assumption is unlikely to have greatly affected the reliability of the results; in fact, it is perhaps more likely that some of the cultivars that were identified as being different clones but have genotype mismatches at two or three loci have originated from the same clones and have been maintained by grafting propagation. If so, their morphological variations presumably derive from bud sport mutations. Alternatively, they could be synonymous cultivars. We might only overlook the absence of apparent morphological differences, particularly since limited morphological field observations were conducted, over a short period of time during blossoming. These possibilities require further investigation.

We chose to study Japan's leading collections of flowering cherry cultivars, which appear to have been managed properly. Nevertheless, many errors were found in the nominal designations in the archived records for the cultivated lines. This suggests that misclassifications and simple management errors commonly occurred during the labeling and transplanting of the trees. Because such erroneous information diminishes the value of the cultivated lines, more careful management will be required in future.

SSR marker analysis provided important insights into the clonal status of the Japanese flowering cherry cultivars. Because it is very difficult to identify cultivars on the basis of morphological observations alone, and the short duration of the flowering season, SSR maskers are expected to be powerful tools for cultivar characterization that will find widespread use in assaying garden trees and newly propagated young saplings. In addition, the current taxonomy of the cultivars of the Japanese flowering cherry should be reconsidered. Our future work will need to address this issue. We intent to devise a new taxonomy through more detailed observation of the morphological characteristics and reconsideration of the previous studies, in light of the genotypic classification reported in this study.

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