Development and characterization of chloroplast simple sequence repeat markers for *Prunus* taxa (eleven Japanese native taxa and two foreign taxa)

Shuri Kato[1, 2], Asako Matsumoto[1], Reiko Mizusawa[3], Yoshiaki Tsuda[4], Yoshihiko Tsumura[5], Hiroshi Yoshimaru[1]

[1] Department of Forest Molecular Genetics and Biotechnology, Forestry and Forest Products Research Institute, 1 Matsuno sato, Tsukuba, Ibaraki 305-8687, Japan
[2] Tama Forest Science Garden, Forestry and Forest Products Research Institute, 1833-81 Todorimachi, Hachioji, Tokyo 193-0843, Japan
[3] Faculty of Human Development and Culture, University of Fukushima, 1 Kanayagawa, Fukushima-shi, Fukushima 960-1296, Japan
[4] Sugadaira Research Station, Mountain Science Center, University of Tsukuba, 1278-294 Sugadairakogen, Ueda, Nagano 386-2204, Japan
[5] Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-8572, Japan

Corresponding author: Shuri Kato, e-mail address: shuri@affrc.go.jp

Abstract

Japanese flowering cherry (*Prunus* subgenus *Cerasus*; Rosaceae) cultivars, which are characterized by beautiful flowers, have been generated through hybridization among wild *Prunus* taxa. The analysis of polymorphisms in the maternally inherited chloroplast DNA is an effective complementary approach for tracing the origins of these cultivars. Thus, a set of novel chloroplast simple sequence repeat (cpSSR) markers was developed for the *Prunus* taxa, and their utility in cross-species amplification was evaluated. Five markers were designed from the partial chloroplast genome sequences of thirteen *Prunus* taxa. In all, we found 19 haplotypes among the 311 individuals tested. The number of haplotypes, haplotype richness and haplotype diversity in each taxon ranged from 1 to 8, from 1 to 6.613 and from 0 to 0.758, respectively. The major proportion of the total diversity (H_T = 0.831) was accounted for by intraspecific diversity (H_S = 0.559), and the level of species subdivision, G_{ST}, was 0.327. These five cpSSR markers highly polymorphic, and they will be useful not only for tracing the origins of the cultivars but also for studying the population structure, diversity, and phylogeography of *Prunus*.

Keywords: : *Prunus, Cerasus, flowering cherry, cpDNA, SSR, genetic diversity*

Introduction

Japanese flowering cherry (*Prunus* subgenus *Cerasus*; Rosaceae) cultivars, which bear beautiful flowers, exhibit extensive variation in the morphological traits, and the taxonomy and genealogy are confusing because of long history of cultivation (Kawasaki 1993, Kuitert 1999). Molecular markers have been used in taxonomic studies of Japanese flowering cherry cultivars, efficiently overcoming the disadvantages of classification systems based on the morphological traits. Recently, the molecular analysis by using the nuclear simple sequence repeat (SSR) markers resulted in improved understanding of the clonal status of numerous cultivars (Kato et al. 2012), and revealed that many cultivars originated from hybrids between two or more wild *Prunus* species (Kato et al. 2014). However, there is still some uncertainty about the taxonomy and genealogy. The uniparentally inherited nature of chloroplast DNA in plants could overcome the disadvantages encountered only in analyses of the biparentally inherited nuclear DNA, and the polymorphic chloroplast SSRs (cpSSRs), characterized by poly (A) or poly (T) mononucleotide repeats have been frequently used as ideal markers for not only cultivar origin and pedigree studies in crop species but also population genetics in wild species (Provan et al. 1999, 2001, Arroyo-Garcia et al. 2002, Wheeler et al. 2014). The variations at the cpSSRs have been also used to study the genetic relationship and the phylogeography in Chinese and European *Prunus* species (Pervaiz et al. 2015, Xuan et al. 2011, Wang et al. 2017). The application of cpSSR markers

DOI:10.2478/sg-2018-0018
edited by the Thünen Institute of Forest Genetics

should provide a powerful complementary method for studying genetic diversity of Japanese flowering cherry cultivars, and could be an effective approach for more detailed tracing their origins, especially the maternal parentage. Thus, a set of novel cpSSR markers was developed for the *Prunus*, and the genetic diversity and structure of thirteen *Prunus* taxa were also evaluated through the testing their utility in cross-species amplification.

Materials and Methods

We sampled thirteen *Prunus* taxa (eleven Japanese taxa; *P. maximowiczii* Ruprecht, *P. pendula* Maxim *f. ascendens* (Makino) Ohwi, *P. apetala* (Sieb. et Zucc.) Franch. et Savat. var. *apetala, P. apetala* (Sieb. et Zucc.) Franch. et Savat. var. *pilosa* (Koidz.) Wilson, *P. incisa* Thunb. ex Murray var. *incisa*, *P. incisa* Thunb. ex Murray var. *kinkiensis* (Koidz.) Ohwi, *P. nipponica* Matsum, *P. sargentii* Rehder, *P. verecunda* (Koidz.) Koehne, *P. jamasakura* Sieb. ex Koidz. and *P. lannesiana* (Carr.) Wilson var. *speciosa* (Koidz.) Makino, and two foreign taxa; *P. campanulata* Maxim. and *P. pseudocerasus* Lindley), which we had previously surveyed (Kato et al. 2014). The cpDNA polymorphisms were screened against a panel comprising one or two individuals randomly selected from each taxon (16 individuals in total). The specific intergenic spacers and introns of cpDNA, which were amplified by using 13 primer pairs listed in Kato et al (2011), were surveyed. The polymerase chain reaction (PCR) amplification and subsequent sequencing also followed the procedure described in Kato et al (2011). The cpDNA regions, which included highly polymorphic SSRs in the screening panel, were used for PCR primer design.

Cross-species testing of novel cpSSR markers was performed on 311 individuals (13 to 37 individuals of each taxon) from thirteen *Prunus* taxa, whose DNA samples have been already prepared in Kato et al. (2014). The cpSSR analysis followed the procedure described in Kato et al. (2012). To assess the informativeness of the markers developed, the polymorphic information content (PIC) was calculated for each marker. The haplotypes were determined by combining information for all polymorphic regions. Genetic diversity indices such as the number of haplotypes (*N*), haplotype richness (*R*), and haplotype diversity (*H*) were calculated for each taxon using FSTAT v. 2.9.3.2 (Goudet 2001). The level of species subdivision (G_{ST}) was also calculated from the total diversity (H_T) and intraspecific diversity (H_S).

Table 1
Characteristics of five polymorphic cpSSR markers for *Prunus*

Marker	Forward primer sequence	Reverse primer sequence	PCR fragment size (bp)	PIC
atpFintron_415†	TTCCCGAACCAAACATGAAT	TTGGATTAGCGATCCGTTTC	154 and 155	0.175
atpFintron_628‡	GAAACGGATCGCTAATCCAA	ACAAATCGGAAAAACGGGTA	193, 194, 195 and 196	0.366
atpFintron_715†	ACCCGTTTTTCCGATTTGTT	GAAGTTCAGATGCAGCATGG	172, 173, 174, 175 and 176	0.353
atpFintron_928‡	TTTGAACCCGCTTCCATATT	CATGATTCCGCGAATTTCTT	213, 214 and 215	0.481
rpL20-rpS12_248†	TATAACCTTCCCGACCACGA	AAAGAAGGGCTCCGGTGTAT	195, 196, 197, 198 and 202	0.323

PIC: polymorphic information content.
PCR reactions were performed using multiple pairs of primers marked by † and ‡, respectively.

Table 2
Occurrence frequencies (%) of chloroplast haplotypes and genetic diversity indices in each *Prunus taxon*

Chloroplast haplotype	max	cam	pse	pen	ape	ape.pil	inc	inc.kin	nip	sar	ver	jam
a (154/193/172/213/197)											2.8 (1)	
b (154/193/173/213/196)	10.5 (2)				8.7 (2)	25.0 (3)	16.7 (2)	71.4 (10)	4.3 (1)	6.1 (2)	5.6 (2)	16.7 (6)
c (154/193/173/214/196)		73.3 (11)			52.2 (12)	41.7 (5)	50.0 (6)	21.4 (3)	60.9 (14)	12.1 (4)	41.7 (15)	2.8 (1)
d (154/193/173/214/197)									4.3 (1)	3.0 (1)		
e (154/193/173/214/202)											5.6 (2)	5.6 (2)
f (154/193/173/215/196)									13.0 (3)	3.0 (1)		
g (154/193/174/213/196)	15.8 (3)						8.3 (1)				5.6 (2)	11.1 (4)
h (154/193/174/215/196)									8.7 (2)			
i (154/193/175/213/196)	57.9 (11)		100.0 (15)		8.7 (2)	8.3 (1)			8.7 (2)	3.0 (1)	5.6 (2)	
j (154/193/176/213/196)	15.8 (3)											
k (154/194/173/213/196)					26.1 (6)	25.0 (3)	16.7 (2)	7.1 (1)		48.5 (16)	27.8 (10)	2.8 (1)
l (154/194/173/213/197)										18.2 (6)		
m (154/194/173/214/196)										6.1 (2)	5.6 (2)	
n (154/195/173/213/195)					4.3 (1)		8.3 (1)					58.3 (21)
o (154/195/173/214/195)												2.8 (1)
p (155/193/173/213/196)		20.0 (3)										
q (155/193/173/213/197)				16.7 (5)								
r (155/193/173/213/198)				83.3 (25)								
s (155/193/173/214/196)		6.7 (1)										
N	4	3	1	2	5	4	5	3	6	8	8	7
R	3.98	2.97	1.00	2.00	4.69	4.00	5.00	2.98	5.46	6.46	6.61	5.45
H	0.637	0.448	0.000	0.287	0.672	0.758	0.742	0.473	0.621	0.729	0.754	0.632

N: number of haplotypes, *R*: haplotype richness, and *H*: haplotype diversity.
Chloroplast haplotypes were determined by combining information for all polymorphic regions. The information of fragment size is shown in order of atpFintron_415, atpFintron_628, atpFintron_715, atpFintron_928 and rpL20-rpS12_248.
More than 0.2 and 0.5 of the occurrence frequencies were *underlined* and *double-underlined*, respectively.
The abbreviated names used for *P. maximowiczii*, *P. campanulata*, *P. pseudocerasus*, *P. pendula* f. *ascendens*, *P. apetala* var. *apetala*, *P. apetala* var. *pilosa*, *P. incisa* var. *incisa*, *P. incisa* var. *kinkiensis*, *P. nipponica*, *P. sargentii*, *P. verecunda*, *P. jamasakura*, *P. lannesiana* var. *speciosa* are max, cam, pse, pen, ape, ape.pil, inc, inc.kin, nip, sar, ver, jam and lan.spe, respectively.

Results and Discussion

Four cpSSR regions in the *atp*F intron (Weising and Gardner 1999) and one in *rpl*20-*rps*12 spacer (Hamilton 1999) were used for PCR primer design (GenBank accession numbers LC327001 and LC327000). The number of polymorphic fragments per marker ranged from 2 to 5, and the polymorphic information content (PIC) value from 0.175 to 0.481 (Table 1). A total of 19 haplotypes were identified by combining information for all five polymorphic regions (Table 2). The number of haplotypes (*N*), haplotype richness (*R*) and haplotype diversity (*H*) in each taxon ranged from 1 to 8, from 1 to 6.613 and from 0 to 0.758, respectively. The major proportion of the total diversity (H_T = 0.831) was accounted for by intraspecific diversity (H_S = 0.559), and the level of species subdivision, G_{ST}, was 0.327. Some haplotypes appeared more frequently in specific taxa. For example, in *P. pendula* f. *ascendens*, no haplotypes were shared with the others. If the other cpSSR regions are explored in more detail, then many more species-specific polymorphisms could be found and the cpSSR analysis would possibly permit the species discrimination in *Prunus*.

The newly developed *Prunus* cpSSR markers could be applied to detect the interspecific and intraspecific genetic variation in chloroplast DNA, and they should prove to be a useful complementary tool for the taxonomic studies. The risk of homoplasy existing in cpSSR regions may limit the application in phylogenetic studies (Weising and Gardner 1999). However, accurate origins and pedigrees of the Japanese flowering cherry cultivars can be deduced by combining the results of previous nuclear DNA markers with those of the present cpSSR markers. Furthermore, as stated by Provan et al. (2001) and Wheeler et al. (2014), these cpSSR markers will be also useful for studying the population structure, diversity, and phylogeography of *Prunus*.

Acknowledgements

This research was supported by a grant for "Research on Genetic Guidelines for Restoration Programs using Genetic Diversity Information" from the Ministry of Environment, Japan and Grant-in-Aid for Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology, Japan, grant numbers: 24380087 and 16K07797. We thank Yuzuru Mukai, Takayuki Kawahara, Toshio Katsuki and Kojiro Iwamoto for their help in sampling the plant material.

References

Arroyo-García R, Lefort F, de Andrés MT, Ibáñaez J, Borrego J, Jouve N, Cabello F, Martínez-Zapater JM (2002). Chloroplast microsatellite polymorphisms in Vitis species. Genome 45: 1142–1149. https://doi.org/10.1139/g02-087

Goudet J (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www2.unil.ch/popgen/softwares/fstat.htm

Hamilton MB (1999). Four primer pairs for the amplification of chloroplast intergenic regions with interspecific variation. Mol. Ecol. 8: 521–523.

Kato S, Iwata H, Tsumura Y, Mukai Y (2011). Genetic structure of island populations of Prunus lannesiana var. speciosa revealed by chloroplast DNA, AFLP and nuclear SSR loci analyses. J. Plant Res. 124: 11–23. https://doi.org/10.1007/s10265-010-0352-3

Kato S, Matsumoto A, Yoshimura K, Katsuki T, Iwamoto K, Tsuda Y, Ishio S, Nakamura K, Moriwaki K, Shiroishi T, Gojobori T, Yoshimaru H (2012). Clone identification in Japanese flowering cherry (Prunus subgenus Cerasus) cultivars using nuclear SSR markers. Breed. Sci. 62: 248–255. https://doi.org/10.1270/jsbbs.62.248

Kato S, Matsumoto A, Yoshimura K, Katsuki T, Iwamoto K, Kawahara T, Mukai Y, Tsuda Y, Ishio S, Nakamura K, Moriwaki K, Shiroishi T, Gojobori T, Yoshimaru H (2014). Origins of Japanese flowering cherry (Prunus subgenus Cerasus) cultivars revealed using nuclear SSR markers. Tree Genet. Genomes 10: 477–487. https://doi.org/10.1007/s11295-014-0697-1

Kawasaki T (1993). Flowering cherries of Japan. Yama-kei Publisher, Tokyo. (In Japanese)

Kuitert W (1999) Japanese flowering cherries. Timber Press, Portland, Oregon, USA.

Pervaiz T, Sun X, Zhang Y, Tao R, Zhang J, Fang J (2015). Association between Chloroplast and Mitochondrial DNA sequences in Chinese Prunus genotypes (Prunus persica, Prunus domestica, and Prunus avium). BMC Plant Biol. 15. 4. https://doi.org/10.1186/s12870-014-0402-4

Provan J, Russell JR, Booth A, Powell W (1999). Polymorphic chloroplast simple sequence repeat primers for systematic and population studies in the genus Hordeum. Mol. Ecol. 8: 505–511. https://doi.org/10.1046/j.1365-294x.1999.00545.x

Provan J, Powell W, Hollingsworth PM (2001). Chloroplast microsatellites: new tools for studies in plant ecology and evolution. Trend. Ecol. Evol. 16: 142-147. https://doi.org/10.1016/s0169-5347(00)02097-8

Wang Z, Zeng Y, Zhang Z, Sheng S, Tian J, Wu R, Pang X (2017). Phylogeography Study of the Siberian Apricot (Prunus sibirica L.) in Northern China Assessed by Chloroplast Microsatellite and DNA Makers. Front Plant Sci. 8:1989. https://doi.org/10.3389/fpls.2017.01989

Weising K, Gardner RC (1999). A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome 42: 9–19. https://doi.org/10.1139/gen-42-1-9

Wheeler GL, Dorman HE, Buchanan A, Challagundla L, Wallace LE (2014). A review of the prevalence, utility, and caveats of using chloroplast simple sequence repeats for studies of plant biology. Appl. Plant Sci. 2: 1400059. https://doi.org/10.3732/apps.1400059

Xuan H, Spann D, Schlottmann P, Neumüller M (2011). Approaches to determine the origin of European plum (Prunus domestica) based on DNA nucleotide sequences. Acta Hortic. 918: 261-267. https://doi.org/10.17660/actahortic.2011.918.32