Maternal Lineages of the Cultivated Strawberry, *Fragaria* × *ananassa*, Revealed by Chloroplast DNA Variation

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Abstract. We analyzed sequence variation in chloroplast DNA (cpDNA) to investigate the origin of the cultivated strawberry, *Fragaria* × *ananassa*. From analysis of two noncoding regions, *trnL-trnF* and *trnR-rn5*, we found three haplotypes (V, C, and X) in *F. ×ananassa*. Haplotype V corresponded to the haplotype of *F. virginiana* and was possessed by cultivars bred over a wide geographic range, including North America, Europe, and Japan. Almost all the North American cultivars analyzed in this study possessed haplotype V, suggesting a founder effect. Haplotype C corresponded to the haplotype of *F. chiloensis* and was detected mainly in Japanese cultivars. Haplotype X was found in only two English cultivars. This haplotype was positioned as intermediate between haplotypes V and C in a median-joining network and was considered to be representative of the process of differentiation between *F. virginiana* and *F. chiloensis*. Results of controlled crosses indicate that cpDNA haplotypes of *F. ×ananassa* are maternally inherited. These results verify that *F. ×ananassa* is an interspecific hybrid between *F. virginiana* and *F. chiloensis* and indicate that traditional cultivars of *F. ×ananassa* have been derived from at least three maternal lineages. We demonstrate that the cpDNA variation detected in this study can be used to verify parentage and for extending hypotheses about June yellows, a leaf variegation disorder in strawberry.

Determining the historical pedigree of cultivars is an important step in understanding the evolution of crop species (Matsuoka, 2005). It can also help to avoid inbreeding depression and to identify the origin of agronomically important traits.

The cultivated strawberry, *Fragaria ×ananassa* Duch., is one of the most economically important fruit crops in the world. It first arose from accidental hybridization between two American octoploid species, *F. virginiana* and *F. chiloensis*, in a European garden during the early to mid-1700s (Darrow, 1966). The number of *F. chiloensis* involved

in that first hybridization was claimed to have been only five plants, which were introduced from Chile to Europe in 1714 (Darrow, 1966). Systematic breeding began in England in the early 1800s and in North America in the mid-1800s using a small number of native and cultivated clones (Darrow, 1966). Most modern strawberry cultivars are the progeny of this relatively narrow range of germplasm, although attempts have been made recently to increase genetic diversity by using wild genetic resources (Hancock et al., 2001; Luby et al., 2008). On the basis of pedigree data, Dale and Sjulín (1990) reported that the majority of modern North American cultivars came from only 17 cytoplasmic sources. However, further tracing was impossible owing to incomplete records.

Because chloroplast DNA (cpDNA) is unaffected by changes in ploidy, which can complicate phylogenetic analysis, the genome is particularly useful for the phylogenetic analysis of *Fragaria*. Potter et al. (2000) examined the sequence variation in cpDNA among 14 species of *Fragaria* with various

Table 1. Country of origin and the detected chloroplast DNA haplotype of 75 accessions of *Fragaria ×ananassa* and seven accessions of its wild relatives.

Accessions	Country	Haplotype
<i>F. ×ananassa</i> cultivars in North America		
Allstar	USA	V
Arking	USA	V
Blakemore	USA	V
Cardinal	USA	V
Chandler	USA	V
Columbia	USA	C
Donner	USA	V
Douglas	USA	V
Excelsior	USA	V
Fairfax	USA	V
Fresno	USA	V
Geneva	USA	V
Hecker	USA	V
Holiday	USA	V
Honeoye	USA	V
Huxley	USA	V
Lassen	USA	V
Linn	USA	V
Marshall	USA	V
Missionary	USA	V
Pajaro	USA	V
Premier (Howard 17)	USA	V
Raritan	USA	V
Selva	USA	V
Sequoia	USA	V
Tioga	USA	V
Tyee	Canada	V
Vibrant	Canada	V
Wiltguard	USA	V
<i>F. ×ananassa</i> cultivars in Europe		
Cambridge Favourite	UK	X
Cambridge Prizewinner	UK	V
Deutch Evern	Germany	V
Merton Princess	UK	X
Oranda	Netherlands	V
Redgauntlet	UK	V
Senga Sengana	Germany	V
<i>F. ×ananassa</i> cultivars in Japan		
Aiberry	Japan	V
Akihime	Japan	V
Asuka Wave	Japan	C
Bellerouge	Japan	V
Benihoppe	Japan	V
Decorouge	Japan	V
Enrai	Japan	V
Everberry	Japan	V
Fukuba	Japan	C
Harunoka	Japan	V
Haruyoi	Japan	V
Hatsukuni	Japan	C
Himiko	Japan	C
Hokowase	Japan	V
Kitanokagayaki	Japan	V
Kogyoku	Japan	V
Kurume 34	Japan	C
Kurume 103	Japan	V
Miyazaki	Japan	V
Mo-ikko	Japan	V
Morioka 16	Japan	V
Morioka 26	Japan	V
Morioka 30	Japan	V
Morioka 32	Japan	V
Morioka 33	Japan	V
Morioka 34	Japan	V
Natsuakari	Japan	V
Nyoho	Japan	V
Ohishi Shikinari	Japan	V
Reiko	Japan	V
Sachinoka	Japan	C
Sagahonoka	Japan	C

(Continued on next page)

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Table 1. (Continued) Country of origin and the detected chloroplast DNA haplotype of 75 accessions of *Fragaria ×ananassa* and seven accessions of its wild relatives.

Accessions	Country	Haplotype
Summer Candy	Japan	C
Summer Princess	Japan	V
Summerberry	Japan	V
Tochihitomi	Japan	C
Tochiotome	Japan	C
Toyonoka	Japan	C
Yachiyo	Japan	V
Wild relatives		
<i>F. virginiana</i> ^a		V
<i>F. virginiana</i> ^b		V2
<i>F. chiloensis</i> 'PI551445'		C
<i>F. vesca</i> EMC		Vesca
<i>F. vesca</i> UC1		Vesca
<i>F. vesca</i> UC5		Vesca
<i>F. nilgerrensis</i> ^a		Nil

^aGermplasm conserved in the National Agricultural Research Center for Tohoku region, Morioka, Japan.

^bSeeds were purchased from the B&T World Seeds, Pagny-sur-Moselle, France, in 2002.

ploidy levels. Distinctive sequences for each species, including *F. virginiana* and *F. chiloensis*, were found in the *trnL-trnF* region, and *F. ×ananassa* showed the same sequence as *F. virginiana*. Although this analysis examined only one sample of *F. ×ananassa* and therefore did not provide further information about the involvement of other wild species in the establishment of *F. ×ananassa*, sequence variation in cpDNA may allow us to infer the origin of each strawberry cultivar.

In this study, we investigated the mode of inheritance of cpDNA and then identified the maternal origin of *F. ×ananassa*. We discuss the accuracy of the reported pedigree of cultivars and the relationship between cpDNA haplotype and June yellows, a leaf variegation disorder in strawberry.

Materials and Methods

We collected fresh leaves from 75 accessions of *F. ×ananassa* and from a total of seven accessions from four related species, *F. virginiana*, *F. chiloensis*, *F. vesca*, and *F. nilgerrensis*, grown at the National Agricultural Research Center for Tohoku Region, one of Japan's official gene banks for strawberries (Table 1). Total DNA was extracted by using a modified PEG method (Rowland and Nguyen, 1993) with Plant DNAzol Reagent (Invitrogen, Carlsbad, CA), as described by Sugimoto et al. (2005). Two noncoding regions of cpDNA were selected for polymerase chain reaction (PCR) amplification, because previous studies revealed that these regions contain intraspecific polymorphisms (Potter et al., 2000; Honjo et al., unpublished data), specifically the spacer between *trnL* and *trnF* and the spacer between *trnR* and *rrn5*. The primers used are listed in Table 2. The PCR reaction mix contained 1 × PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 100 μM each dNTP, 0.02% Triton X-100, 0.01% gelatin], 1.5 mM

Table 2. Chloroplast DNA primers used in the analysis of *Fragaria ×ananassa*, annealing temperature, and references.

Primer	Sequence 5'-3'	Annealing temp (°C)	References
<i>trnL</i> (UAA)	GGTTCAAGTCCCTCTATCCC	50	Taberlet et al. (1991)
<i>trnF</i> (GAA)	ATTTGAAGTGGTGACACGAG	50	Taberlet et al. (1991)
<i>trnR</i> (ACG)	CGACACCGTGGTTCGTAGC		Yoshimura et al. (unpublished data)
<i>rrn5</i>	TGGTGTCCCAGGCGTAGAG		Yoshimura et al. (unpublished data)

MgCl₂, 0.9 units Taq polymerase, 0.2 μM of each primer, and 10 ng template DNA in a total volume of 30 μL. Thermocycling conditions were as follows: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C; and a final extension step at 72 °C for 5 min. The PCR was carried out in a GeneAmp PCR System Model 9700 (Applied Biosystems, Foster City, CA) or a PCR Thermal Cycler Dice (Takara, Tokyo, Japan). The PCR products were purified with a QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany). The DNA obtained was sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) using a Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequencing data were aligned manually with CLUSTAL W (Thompson et al., 1994). Insertions/deletions (indels) were generally placed so as to increase the number of matching nucleotides in a sequence position. We determined cpDNA haplotypes from nucleotide substitutions and indels. To show the relatedness of haplotypes, we constructed a median-joining network (Bandelt et al., 1999) with epsilon value set to zero using the software Network 4.5.0.0 (<http://www.fluxus-engineering.com>).

To confirm the mode of inheritance of cpDNA in *F. ×ananassa*, we performed controlled cross-pollination between cultivars possessing different haplotypes (Table 3). We examined the haplotype for one to five seedlings per cross.

Results

We found two haplotypes (V and V2) in *F. virginiana* that differed only in the number of tandem repeats of mononucleotides in the intergenic spacer between *trnR* and *rrn5* (Tables 1 and 4). *Fragaria chiloensis*, *F. vesca*, and *F. nilgerrensis* possessed the distinct haplotypes C, vesca, and nil, respectively. In *F. ×ananassa*, we detected three haplotypes (V, C, and X). Haplotype X displayed the same sequence as haplotype C in the spacer between *trnL* and *trnF* and as haplotype V in the spacer between *trnR* and *rrn5*. The median-joining network analysis placed haplotype X between haplotypes V and C (Fig. 1). The nucleotide sequences of these haplotypes will appear in the DNA Data Bank of Japan under accession numbers AB514801 to AB514816.

We found haplotype V in 61 cultivars of *F. ×ananassa* originating from a diverse geographic range, namely, North America, Europe, and Japan. All North American cultivars except 'Columbia' possessed haplotype V. We found haplotype C in 11

Table 3. Chloroplast DNA haplotype of seedlings obtained from controlled cross-pollination of cultivars of *Fragaria ×ananassa*.

Parentage (female × male)	Haplotype
Haplotype C × haplotype V	
Sagahonoka × Natsuakari, No.1	C
Sagahonoka × Natsuakari, No.2	C
Sagahonoka × Natsuakari, No.3	C
Sagahonoka × Natsuakari, No.4	C
Sagahonoka × Natsuakari, No.5	C
Sagahonoka × Selva, No.1	C
Sagahonoka × Selva, No.2	C
Haplotype V × haplotype C	
Natsuakari × Sagahonoka, No.1	V
Natsuakari × Sagahonoka, No.2	V
Natsuakari × Sagahonoka, No.3	V
Selva × Sagahonoka	V
Haplotype V × haplotype X (Morioka 32 × Sagahonoka)	
× Cambridge Favourite	V
Haplotype X × haplotype V	
Cambridge Favourite × Aiberry	X
Cambridge Favourite × Kitanokagayaki	X
Cambridge Favourite × Morioka 32	X
Haplotype X × haplotype C	
Cambridge Favourite × Sachinoka	X

Japanese cultivars and the American cultivar Columbia. We detected haplotype X in only two English cultivars, Cambridge Favourite and Merton Princess.

All seedlings obtained from controlled cross-pollination possessed the same haplotypes as their maternal parents (Table 3).

Discussion

The results of the controlled crosses (Table 3) indicate that the cpDNA of *F. ×ananassa* is maternally inherited, as it is in many angiosperms, including other Rosaceae genera such as *Prunus* (Bouhadida et al., 2007), *Malus* (Matsumoto et al., 1997), and *Rubus* (Moore, 1993).

Haplotypes V and C correspond to the haplotypes of *F. virginiana* and *F. chiloensis*, respectively (Table 1). Furthermore, the sequences of the *trnL-trnF* region of each haplotype also corresponded to the sequences of most of *F. virginiana* and *F. chiloensis* accessions analyzed by Potter et al. (2000), respectively. Six of the seven accessions of *F. virginiana* analyzed by Potter et al. (2000) corresponded to haplotype V in our study and one to haplotype C. Also, six of the seven accessions of *F. chiloensis* corresponded to haplotype C and one to haplotype V. Three of the seven accessions of the diploid species *F. vesca* also corresponded to haplotype V. Thus, *F. virginiana* predominantly possesses haplotype V and *F. chiloensis* haplotype C. Haplotype V can be regarded as more

Table 4. Substitutions, indels and repeat variation in *trnL-trnF* and *trnR-rn5* regions of chloroplast DNA in some *Fragaria* species.

Haplotype	Gene region/sequence position							
	<i>trnL-trnF</i>				<i>trnR-rn5</i>			
	100	117	126	186–188	266–272	433	162–171	173 180
V	C	T	A	—	—	T	AAAAAAAAAA	G G
V2	C	T	A	—	—	T	AAAAAAAAAA	G G
C	C	T	A	—	—	C	AAAAAAAAAA	T G
X	C	T	A	—	—	C	AAAAAAAAAA	G G
Vesca	C	T	A	—	CATATCA	T	AAAAAAAAAA	G G
Nil	T	C	C	ATA	—	T	AAAAAAAAAA	G T

Fig. 1. Median-joining network of the chloroplast DNA haplotypes of *Fragaria xananassa* (C, V, X), *F. virginiana* (V, V2), *F. chiloensis* (C), *F. vesca* (vesca), and *F. nilgerrensis* (nil) based on the sequences of two noncoding regions. Each letter corresponds to a haplotype, and the size of the circle is proportional to the haplotype's frequency.

ancestral than haplotype C judging from the relationship with *F. vesca*. The accession that contained haplotype C despite being classified as *F. virginiana* was considered to be the result of introgression, and the accession that contained haplotype V despite being classified as *F. chiloensis* was inferred to be originated from a population that may have been established earlier in the evolution of the two species (Potter et al., 2000). Although haplotype X was not found in wild species, the median-joining network suggests that plants belonging to haplotype X could be classified as either *F. virginiana* or *F. chiloensis* (Fig. 1). The sequence of haplotype X appears to reflect an intermediate point in the process of differentiation between *F. virginiana* and *F. chiloensis*. These results confirm that *F. xananassa* is an interspecific hybrid of *F. virginiana* and *F. chiloensis* (Darrow, 1966) and indicate that traditional cultivars of *F. xananassa* derive from at least three maternal lineages.

Haplotype V is widely distributed, and appears to be predominant in North America (Table 1). Dale and Sjulín (1990) reported that 134 cultivars released in North America between 1960 and 1987 were derived from, at most, 17 maternal founding clones. Of those inferred 17 lineages, three were identified their native clones from pedigree data. Our results support that pedigree: 'Columbia', which was recorded to be derived from *F. chiloensis* 'Reedsport', possessed haplotype C, and 'Arking' and 'Cardinal', which were derived from *F. virginiana* 'The Native Iowa', possessed haplotype V. Although the other 14 founding clones were impossible to trace further on the basis of the pedigree data, our results suggest that nine of these 14 originated from *F. virginiana*, because either

they or their progeny possessed haplotype V (Table 1). The nine are 'Missionary', 'Marshall', 'Hudson Bay' (ancestor of 'Sequoia' and 'Wiltguard'), 'Middlefield' (ancestor of 'Chandler', 'Douglas', 'Hecker', and 'Selva'), 'Chesapeake' (ancestor of 'Allstar' and 'Linn'), 'Aberdeen' (ancestor of 'Holiday' and 'Raritan'), 'Neunan' (ancestor of 'Pajaro' and 'Tyee'), 'Ettersburg 450' (ancestor of 'Honeoye' and 'Vibrant'), and 'Streamliner' (ancestor of 'Geneva'). It is apparent that there has been a strong founder effect in the spread of haplotype V in North America.

Of the Japanese cultivars analyzed in this study, 11 (28%) possessed haplotype C (Table 1), indicating that cultivars with *F. chiloensis* maternal origin are more common in Japan than in North America. Strawberry breeding in Japan began with the establishment of the cultivar Fukuba in 1899. We found that 'Fukuba' possesses haplotype C, which probably explains the relatively high frequency of haplotype C in Japanese cultivars, because its lineage has frequently been used for breeding in Japan.

We found haplotype X in only two cultivars, Cambridge Favourite and Merton Princess (Table 1). Both cultivars were bred in England, 'Cambridge Favourite' in 1947 from a cross between ('Etter seedling' × 'Avant Tout') × 'Blakemore' and 'Merton Princess' in 1956 to 1957 (Darrow, 1966). This suggests that presumably few individuals possessing haplotype X were collected and transferred to England and used for breeding.

Chloroplast DNA variation can be used to verify maternal parentage. 'Kurume 34' is a valuable accession because it is the source of several commercially important Japanese cultivars, including 'Toyonoka', 'Sachinoka', and 'Tochiotome' (presently the most commonly grown cultivar in Japan). 'Kurume 34' is long believed to have been bred from 'Yachiyo' × 'Donner' (Honda et al., 1976). However, we found that 'Kurume 34' and its descendants possess haplotype C, whereas both 'Yachiyo' and 'Donner' possess haplotype V (Table 1; Fig. 2). This suggests that some of the flagship cultivars in Japan are descended from different maternal origin from what has hitherto been reported.

Although the contribution of cytoplasmic genes to agronomically important traits of the strawberry is not well understood, several studies have implicated the cytoplasmic or chloroplast genome in June yellows (Hughes, 1989; Jeong et al., 1988; Rose, 1992), which is a progressive and deleterious chlorosis of

Fig. 2. Reported maternal pedigree of strawberry cultivars leading to Japanese flagship cultivars Tochiotome and Sachinoka. The numbers indicate the year released and the bold letters indicate the chloroplast DNA haplotype of the cultivars. Bold and thin lines connecting accessions indicate a relationship between mother and progeny and that between grandmother and grandchild, respectively.

strawberry leaves (Darrow, 1966; Jamieson and Sanford, 1996). Jeong et al. (1988) and Rose (1992) suggested that inheritance of shade-adapted chloroplasts from *F. virginiana* is one of the possible causes of June yellows. Our study included a number of cultivars in which June yellows has occurred (Hughes, 1989): 'Howard 17 (Premier)', 'Blakemore', 'Pajaro', 'Senga Sengana', 'Tyee', and 'Cambridge Favourite'. All but one possessed haplotype V, which originates from *F. virginiana*. The exception, 'Cambridge Favourite', possessed haplotype X. In contrast, June yellows has so far not been reported from cultivars possessing haplotype C in Japan, although seedlings related to 'Howard 17 (Premier)' developed the condition, just as they do in North America and Europe (Hanaoka et al., 1964). These factors appear to support the arguments of Jeong et al. (1988) and Rose (1992). Also, Rose (1992) raised the possibility that June yellows is an example of hybrid variegation that arises after interspecific crosses in plants with biparental plastid inheritance, resulting in a nonharmonious interaction between the plastids of one parent and the hybrid genome after the "sorting out" of plastids that are compatible. However, we obtained no evidence of cpDNA inheritance from the male parent to progeny. Further analysis of the chloroplast and nuclear genes that play a major role in plastome-genome incompatibility (Yao and Cohen 2000) may

lead to the identification of the mechanism underlying June yellows.

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