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–rrn5*, 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 Sjunin (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 |
|--------------------|---------|-----------|
| *F. ×ananassa* 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         |
| *F. ×ananassa* cultivars in Europe |         |           |
| Cambridge Favourite| UK      | X         |
| Cambridge Prizewinner| UK     | V         |
| Deuth Evern        | Germany | V         |
| Merton Princess    | UK      | X         |
| Oranda             | Netherlands | V    |
| Redgantlet         | UK      | V         |
| Senga Sengana      | Germany | V         |
| *F. ×ananassa* cultivars in Japan |         |           |
| Aiberry            | Japan   | V         |
| Akihime            | Japan   | V         |
| Asuka Wave         | Japan   | C         |
| Bellerouge         | Japan   | V         |
| Benhoppe           | 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         |
| Kitakonagayaki     | 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         |
| Natsukari          | Japan   | V         |
| Nyoho              | Japan   | V         |
| Ohishi Shikinari   | Japan   | V         |
| Reiko              | Japan   | Y         |
| Sachinoka          | Japan   | C         |
| Sagahonoka         | Japan   | C         |

(Continued on next page)
Table 1. (Continued) Country of origin and the detected chloroplast DNA haplotype of 75 accessions of Fragaria virginiana and seven accessions of its wild relatives.

| Accessions     | Country | Haplotype |
|----------------|---------|-----------|
| Summer Candy   | Japan   | C         |
| Summer Princess| Japan   | V         |
| Summerberry    | Japan   | V         |
| Tochihimotsu  | Japan   | V         |
| Tochiotome     | Japan   | C         |
| Toyonoka       | Japan   | C         |
| Yachiyo        | Japan   | V         |

Wild relatives

- F. virginiana* V
- F. virginiana* V2
- F. chiloensis *P551445*
- F. vesca EMC Vesca
- F. vesca UC1 Vesca
- F. vesca UC5 Vesca
- F. nilgerrensis* Nil

*Germplasm conserved in the National Agricultural Research Center for Tohoku region, Morioka, Japan.

*Seeds were purchased from the B&T World Seeds, Pauaguay, France, in 2002.

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

| Primer          | Sequence 5'-3' | Annealing temp (°C) | References                |
|-----------------|---------------|---------------------|----------------------------|
| trnL (UAA)      | GGTCAAGTCCCTCTATCCC | 50                | Taberlet et al. (1991)    |
| trnF (GAA)      | ATTTGAGTTGAGCACGAG | 50                | Taberlet et al. (1991)    |
| trnR (ACG)      | CGACCCGTTCTGCTAGC | 50                | Yoshimura et al. (unpublished data) |
| rrs5            | TGGTGTCACCCGGCGTAGG | 50                | Yoshimura et al. (unpublished data) |

Materials and Methods

We collected fresh leaves from 75 accessions of F. virginiana 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 rrs5. The primers used are listed in Table 2. The PCR reaction mix contained 1 μL PCR buffer [10 μM Tris-HCl (pH 8.3), 50 mM KCl, 100 μM each dNTP, 0.02% Triton X-100, 0.01% gelatin], 1.5 mM 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 BigDye 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. virginiana, 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 rrs5 (Tables 1 and 4). Figuraria chiloensis, F. vesca, and F. nilgerrensis possessed the distinct haplotypes C, vesca, and nil, respectively. In F. virginiana, 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 rrs5. 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. virginiana 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 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. virginiana 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).

Haplotype 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 3. Chloroplast DNA haplotype of seedlings obtained from controlled cross-pollination of cultivars of Fragaria virginiana.

| Parentage (female × male) | Haplotype |
|---------------------------|-----------|
| Haplotypes 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         |
| Haplotypes V × haplotype C|           |
| Natsuakari × Sagahonoka, No.1 | V         |
| Natsuakari × Sagahonoka, No.2 | V         |
| Natsuakari × Sagahonoka, No.3 | V         |
| Selva × Sagahonoka | V         |
| Haplotypes V × haplotype X|           |
| Morioka 32 × Sagahonoka | X         |
| Cambridge Favourite × Aiberry | X        |
| Cambridge Favourite × Kitanokagayaki | X       |
| Cambridge Favourite × Morioka 32 | X       |
| Haplotypes X × haplotype C|           |
| Cambridge Favourite × Sachinoka | X       |
ancestral than haplotype C judging from the Fig. 1. Median-joining network of the chloroplast Table 4. Substitutions, indels and repeat variation in chloroplast genome in June yellows (Hughes, 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. xananassan is an interspecific hybrid of F. virginiana and F. chiloensis (Darrow, 1966) and indicate that traditional cultivars of F. xananassan derive from at least three maternal lineages.

Haplotype V is widely distributed, and appears to be predominant in North America (Table 1). Dale and Sjulin (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 ‘Reedport’, 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 ‘Sequioa’ and ‘Wiltguard’), ‘Middlefield’ (ancestor of ‘Chandler’, ‘Douglas’, ‘Hecker’, and ‘Selva’), ‘Chesapeake’ (ancestor of ‘Allstar’ and ‘Linn’), ‘Aberdeen’ (ancestor of ‘Holiday’ and ‘Raritan’), ‘Neuman’ (ancestor of ‘Pajaro’ and ‘Tyee’), ‘Ettersburg 450’ (ancestor ‘Honoye’ 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.

The accession of ‘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. Although most of the chloroplast DNA variation can be used to verify parental 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 chloroplast DNA variation to the 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 compatibility (Yao and Cohen 2000) may
lead to the identification of the mechanism underlying June yellows.

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