

Karyotype Analysis in Wild Diploid, Tetraploid and Hexaploid Strawberries, *Fragaria* (Rosaceae)

Preeda Nathewet^{1,*}, Tomohiro Yanagi¹, Kim E. Hummer²,
Yoshikane Iwatsubo³ and Kazuyoshi Sone⁴

¹ Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho,
Kita-gun, Kagawa 761-0795, Japan

² USDA ARS National Clonal Germplasm Repository, 33447 Peoria Road,
Corvallis, Oregon 97333-2521, USA

³ Faculty of Science, Department of Biology, Toyama University,
930 Gofuku, Toyama 930-8555, Japan

⁴ Kurume Branch, National Agricultural Research Center for Kyushu
Okinawa Region, 1823, Oi-cho, Kurume, Fukuoka 839-8503, Japan

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Summary The strawberry, genus *Fragaria* (Rosaceae), has a basic chromosome of $x=7$, and is comprised of 20 wild species having an euploid series from diploid ($2n=2x=14$) through decaploid ($2n=10x=70$). Few karyotypes of species in this genus have been reported. The objective of this research was to determine the chromosomal morphology and karyotype analysis of wild diploid, tetraploid and hexaploid *Fragaria* species. Somatic chromosome images of 20 genotypes of diploids, tetraploids, and hexaploids were taken at the metaphase stage of mitosis under a light microscope. Karyotype analysis was performed in 17 accessions. The phylogenetic relationships between species were constructed using cluster analysis based on karyotypic similarity. Somatic chromosome numbers of wild diploid, tetraploid and hexaploid species were $2n=2x=14$, $2n=4x=28$ and $2n=6x=42$. Chromosome morphology in wild diploid species had greater uniformity than that in the tetraploid species. Results of the cluster analysis showed that the diploid and tetraploid species reside in separate clades, with one exception. *Fragaria tibetica*, a tetraploid, clustered with the diploid species clade. The hexaploid, *F. moschata*, clustered with the tetraploid species clade.

Key words Chromosome, *Fragaria*, Karyotype, Polyploidy, Rosaceae, Wild strawberry.

The genus *Fragaria* L. is comprised of about 22 species with varying ploidy levels (Staudt 1989, 1999). The additional taxon is the main cultivated strawberry, *F. ×ananassa* ($8x$), which originated from the spontaneous hybridization between 2 wild octoploid strawberry species, *F. chiloensis* and *F. virginiana*. Wild strawberry species are distributed through the temperate zone of Asia, Europe, North and South America. All species are restricted to a single continent or specific area, except for diploid *F. vesca*, which is found in both Eurasia and America (Staudt 1989, 1999). Twelve diploid species (including a hybrid species) are distributed in Asia, Europe and Western Siberia (Staudt 1999, 2003, 2005, 2006, Staudt and Dickoré 2001, Staudt *et al.* 2003, Staudt and Olbricht 2008). Five tetraploids are restricted to Eastern and South Eastern Asia (Staudt 2003, Staudt and Dickoré 2001), while hexaploid species occur in Europe and Western Siberia (Staudt 1989). Two wild octoploids, *F. chiloensis* and *F. virginiana*, are present in America with distribution in North and South America (Staudt 1989, 1999) while a decaploid, *F. iturupensis*, is endemic to Iturup in the Kuril Islands, Russia (Hummer *et al.* 2009).

The cytological study of strawberry plants had been carried out by Ichijima (1926) and Longley (1926). They observed that the chromosome numbers of *F. vesca* L. were diploid

* Corresponding author, e-mail: pdngw@yahoo.com

Table 1. A list of the taxa used in the present study, assigned to species and summary of their origins

Species	PI number*	Origin
Diploid species $2n=14$		
<i>Fragaria</i> × <i>bifera</i>	616613	Besacon, France
<i>Fragaria bucharica</i>	551851	Nathiagali, Pakistan
<i>Fragaria chinensis</i>	551576	Hubei, China
<i>Fragaria manchurica</i>	CFRA 1947	Unknown
<i>Fragaria nilgerrensis</i>	637950	Yunan, China
<i>Fragaria pentaphylla</i>	651570	Unknown
<i>Fragaria vesca</i> subsp. <i>americana</i>	552286	New York, USA
<i>Fragaria vesca</i> subsp. <i>americana</i>	552287	New Hampshire, USA
<i>Fragaria vesca</i> subsp. <i>bracteata</i>	637947	New Mexico, USA
<i>Fragaria vesca</i> subsp. <i>californica</i>	551513	California, USA
<i>Fragaria vesca</i> subsp. <i>vesca</i>	551826	Europe
<i>Fragaria vesca</i> subsp. <i>vesca</i>	637943	Kotayk Marz, Armenia
<i>Fragaria vesca</i> subsp. <i>vesca</i> f. <i>roseiflora</i>	616872	Louisiana, USA
<i>Fragaria viridis</i>	616857	Gotland, Sweden
<i>Fragaria viridis</i>	616609	Kaiserstuhl, Germany
Tetraploid species $2n=28$		
<i>Fragaria corymbosa</i>	CFRA 1911	Qinghai, China
<i>Fragaria orientalis</i>	602942	Jili, China
<i>Fragaria orientalis</i>	551864	Russia Federation**
<i>Fragaria tibetica</i>	651567	Unknown
<i>Fragaria tibetica</i>	CFRA 1908	Qing Hai, China
Hexaploid species $2n=42$		
<i>Fragaria moschata</i>	551550	Unknown

* all accessions are maintained at NCGR, Corvallis, USA.

** Now classified as "F. hybrid" by NCGR due to the questionable pedigree.

($2n=x=14$), *F. moschata* Weston, hexaploid ($2n=6x=42$), and *F. chiloensis* (L.) Miller and *F. virginiana* Miller were octoploid ($2n=8x=56$). In the same year, Ichijima (1926) determined the basic chromosome numbers of the genus *Fragaria* as $x=7$, with 4 main ploidy levels ranging for diploid ($2n=2x=14$), tetraploid ($2n=4x=28$), hexaploid ($2n=6x=42$), and octoploid ($2n=8x=56$). Subsequently, observations of chromosomes of wild and cultivated strawberry plants have been conducted to confirm the ploidy level (Longley 1926, Petrov *et al.* 1962, Bringhurst and Khan 1963, Bringhurst and Gill 1970, Bringhurst and Senanayake 1966, Hummer *et al.* 2009, Kafkas *et al.* 2002, Nathewet *et al.* 2007, Owen and Miller 1993, Yamaguchi 1980, Yarnell 1929, 1931a, b). Cytologists have also studied the pollen mother cell of strawberry plants to classify the phylogenetic relationship between parent and progeny and genome compositions (Fedorova 1934, Ibrahim *et al.* 1981, Kihara 1930, Scott 1950, Senanayake and Bringhurst 1967, Staudt *et al.* 2003, Yarnell 1931b). Furthermore, karyotype analyses have been conducted on 6 wild diploid species, *F. daltoniana* J. Gay, *F. hayatai* Makino, *F. iinumae* Makino, *F. nipponica* Makino, *F. nubicola* (Hook. f.) Lindl. ex Lacaite, and *F. vesca* L., to describe the karyotypic differences among these species (Iwatsubo and Naruhashi 1989, 1991, Naruhashi *et al.* 1999, Lim 2000, Nathewet *et al.* 2009). Nathewet *et al.* (2009) recently exhibited 56 chromosomes one by one in a wild octoploid plant of *F. chiloensis*.

To date, a great number of studies using morphological and molecular biological data have been performed to clarify the genetic relationship among some *Fragaria* species including wild diploids, tetraploids, hexaploids, and octoploids (Catling and Porebski 1998, Hadonou *et al.* 2004, Harrison *et al.* 1997, Hokanson *et al.* 1993, Hokanson *et al.* 2006, Potter *et al.* 2000). However, few papers have reported karyotype analysis of diploid, tetraploid, or hexaploid strawberry plants, or

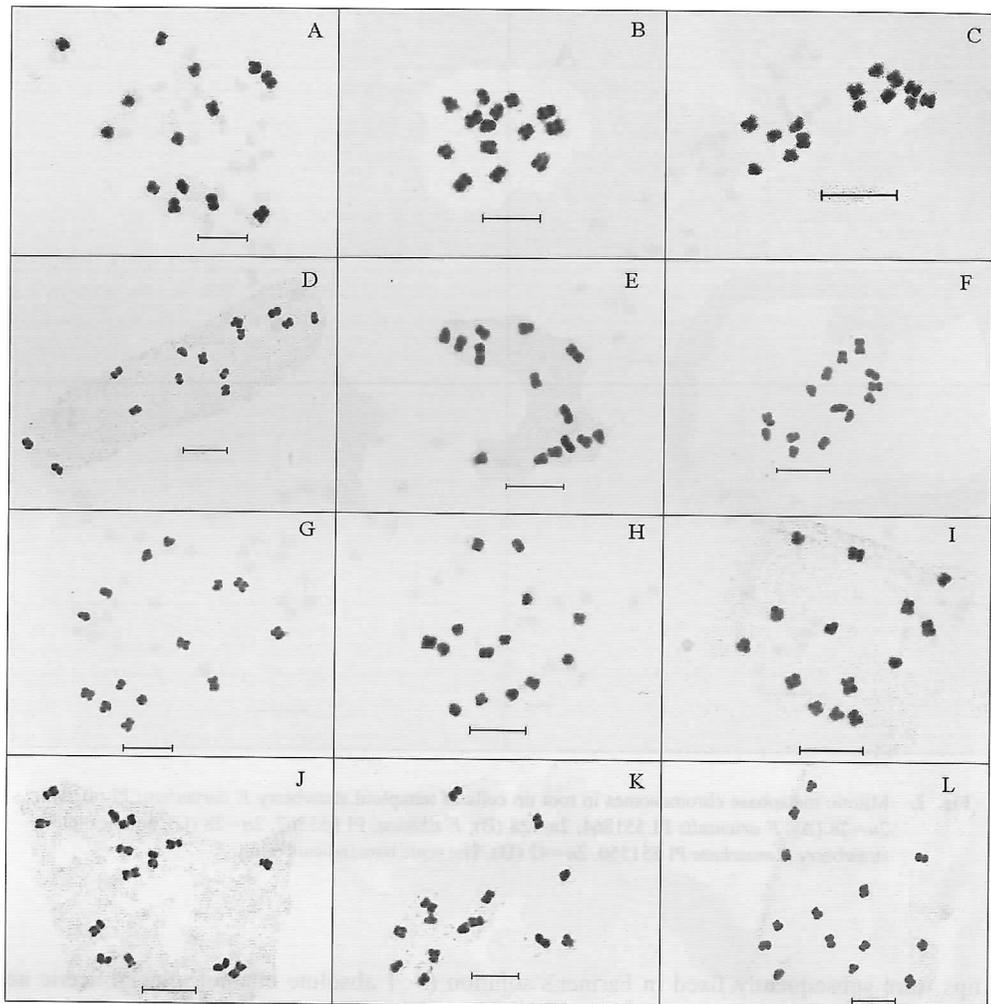


Fig. 1. Somatic chromosomes at the metaphase stage in root tip cells of wild diploid strawberries, all species with $2n=14$. (A) *F. × bifera* PI 616613. (B) *F. bucharica* PI 551851. (C) *F. gracilis* PI ? (CFRA202). (D) *F. mandshurica*, CFRA 1947. (E) *F. nilgerrensis* PI 637950. (F) *F. pentaphylla* PI 651570. (G) *F. vesca* subsp. *americana* PI 552287. (H) *F. vesca* subsp. *bracteata* PI 637947. (I) *F. vesca* subsp. *californica* PI 551513. (J) *F. vesca* subsp. *vesca* PI 551826. (K) *F. vesca* subsp. *roseiflora* PI 616872. (L) *F. viridis* PI 616609. The scale bars indicate 5 μm .

have explored the relationship among them through karyotypic analyses. The objective of this study was to compare the karyotype features among diploid, tetraploid, and hexaploid wild strawberry species, and to propose a phylogeny for them based on karyotypic similarity.

Materials and methods

The somatic chromosomes of 22 genotypes of diploid, tetraploid, and hexaploid wild strawberry species (Table 1) maintained at USDA, ARS, National Clonal Germplasm Repository (NCGR) Corvallis, Oregon, USA, were observed. Root tips were harvested and pretreated with 2 mM 8-hydroxyquinoline at room temperature (22°C) for 1 h and then kept at 4°C for 15 h. The

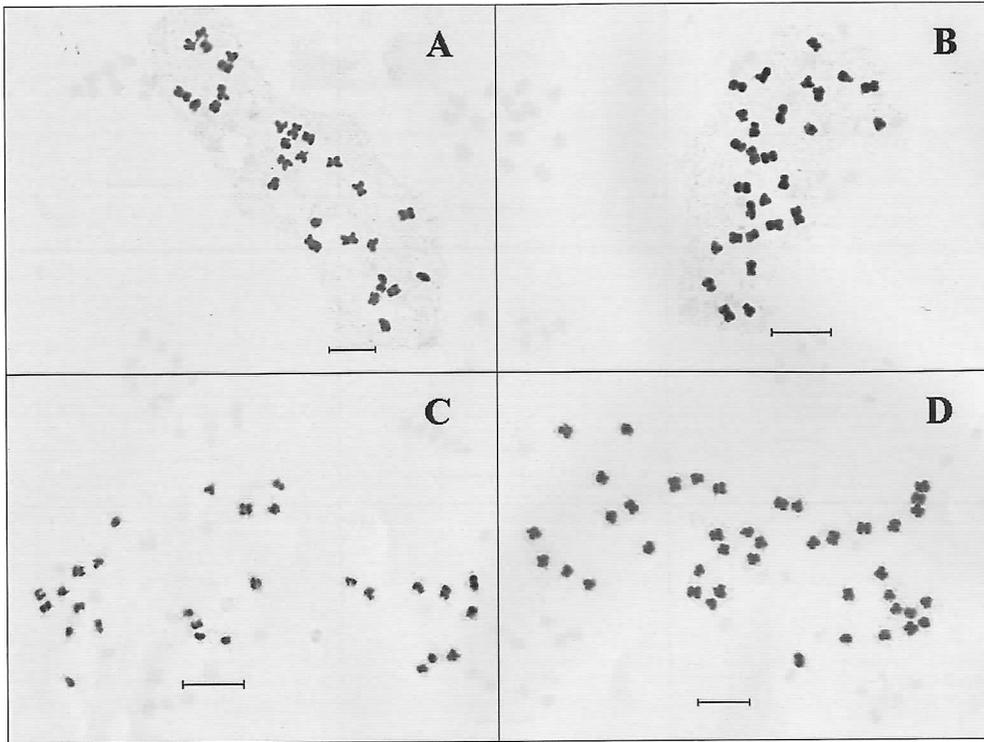


Fig. 2. Mitotic metaphase chromosomes in root tip cells of tetraploid strawberry *F. corymbosa* PI 602942, $2n=28$ (A), *F. orientalis* PI 551864, $2n=28$ (B), *F. tibetica*, PI 651567, $2n=28$ (C), and hexaploid strawberry *F. moschata* PI 551550, $2n=42$ (D). The scale bars indicate $5\ \mu\text{m}$.

root tips were subsequently fixed in Farmer's solution (3 : 1 absolute ethanol: glacial acetic acid), soaked in 1 N HCl at 22°C for 1 h, macerated in 1 N HCl at 60°C for 11 min, and rinsed twice with distilled water. Root tips were stained and squashed with 1.5% lacto-propionic orcein solution. That procedure was similar to that used by Iwatsubo and Naruhashi (1989, 1991) and by Nathewet *et al.* (2007, 2009). Chromosomes were observed and photographed at $100\times$ objective using a light microscope (BX51: Olympus Corp., Tokyo, Japan) equipped with a 3CCD camera (XD500: Olympus Corp., Tokyo, Japan).

For each genotype, at least 10 clear images of somatic cells at the metaphase stage were used to count the chromosomes. Then, for each genotype, 4 of the best images were selected for measuring the long and short arm length. The respective lengths of the long arm (La) and short arm (Sa) of each chromosome were measured using software (FLVFS-FIS: Flovel Co. Ltd., Tokyo, Japan). The nomenclature used for description of chromosome morphology was proposed by Levan *et al.* (1964). Metacentric (m), sub-metacentric (sm) and sub-telocentric (st) chromosomes were noted. As suggested by Levan *et al.* (1964), the following parameters were estimated in each metaphase image to characterize the karyotype numerically: (1) total chromosome length ($\text{TCL} = \sum\text{La} + \sum\text{Sa}$); (2) arm ratio ($\text{AR} = \sum\text{La} / \sum\text{Sa}$); (3) mean haploid chromosome length (MCL) and centromeric index ($\text{CI} = 100 \times \sum\text{Sa} / (\sum\text{La} + \sum\text{Sa})$). A cluster analysis using Ward's method in JMP software (SAS Inc.) was conducted using karyotype numerical data. Variations for HCL, MCL, and CI among species were verified using one-way analysis of variance. Comparisons of means were made using the Tukey-Kramer HSD test at $P < 0.05$.

Table 2. Chromosome numbers, karyotype formulae, mean chromosome length (MCL), total chromosome length (TCL), centromeric index (CI). Chromosome abbreviations: m=metacentric, sm= submetacentric, and st=subtelocentric.

Species	PI number	Chro. No.	Karyotype formulae	Range (mm)	TCL (μm)	MCL (mm)	CI %
<i>Fragaria bucharica</i>	551851	14	8m+6sm	0.89–1.45	17.00 e	1.21 abc	38.31 a
<i>Fragaria</i> × <i>bifera</i>	616613	14	8m+6sm	0.89–1.33	15.63 e	1.12 abcd	37.09 a
<i>Fragaria chinensis</i>	551576	14	8m+6sm	0.61–0.93	11.00 f	0.79 e	35.86 a
<i>Fragaria manchurica</i>	CFRA 1947	14	10m+4sm	0.91–1.40	15.76 e	1.13 abcd	38.54 a
<i>Fragaria nilgerrensis</i>	637950	14	8m+6sm	1.12–1.60	18.29 e	1.31 a	37.70 a
<i>Fragaria pentaphylla</i>	651570	14	10m+2sm+2st	0.97–1.49	16.51 e	1.18 abc	37.32 a
<i>Fragaria vesca</i> subsp. <i>americana</i>	552287	14	10m+4sm	0.82–1.24	14.89 ef	1.06 bcd	39.50 a
<i>Fragaria vesca</i> subsp. <i>bracteata</i>	637947	14	10m+4sm	0.84–1.20	14.48 ef	1.03 bcd	38.50 a
<i>Fragaria vesca</i> subsp. <i>californica</i>	551513	14	10m+4sm	0.86–1.20	14.25 ef	1.02 cde	38.79 a
<i>Fragaria vesca</i> subsp. <i>vesca</i>	551826	14	8m+4sm+2st	0.80–1.27	14.58 ef	1.04 bcd	37.42 a
<i>Fragaria vesca</i> subsp. <i>vesca</i> f. <i>roseiflora</i>	616872	14	8m+4sm+2st	0.97–1.36	16.36 e	1.17 abc	38.34 a
<i>Fragaria viridis</i>	616609	14	8m+6sm	0.87–1.28	15.19 ef	1.09 abcd	38.18 a
<i>Fragaria corymbosa</i>	CFRA 1911	14	16m+12sm	0.77–1.36	28.69 cd	1.02 cde	37.91 a
<i>Fragaria orientalis</i>	602942	28	12m+16sm	0.93–1.85	36.26 b	1.29 ab	35.21 a
<i>Fragaria orientalis</i>	551864	28	14m+14sm	0.93–1.57	31.35 bc	1.13 abc	39.08 a
<i>Fragaria tibetica</i>	651567	28	18m+10sm	0.69–1.15	24.99 d	0.89 de	37.48 a
<i>Fragaria moschata</i>	551550	42	30m+10sm+2st	0.83–1.27	43.54 a	1.04 bcd	38.85 a
<i>F</i> (ANOVA) $P=0.05$					100.8671	8.2418	1.6616
P value					<0.0001	<0.0001	<0.0842
<i>Fragaria vesca</i> subsp. <i>americana</i>	552286	14	—	—	—	—	—
<i>Fragaria vesca</i> subsp. <i>vesca</i>	637943	14	—	—	—	—	—
<i>Fragaria viridis</i>	616857	14	—	—	—	—	—
<i>Fragaria tibetica</i>	CFRA 1908	28	—	—	—	—	—

Note: Values in the same column with different letters are significantly different.

Results

Somatic chromosome images at the metaphase stage of the studied taxa are shown (Figs. 1, 2). The chromosome number for each accession is presented (Table 2). The somatic chromosome number of all wild diploid, tetraploid and hexaploid strawberries is $2n=2x=14$, $2n=4x=28$, and $2n=6x=42$, respectively.

The karyotype formulae obtained and parameters analyzed for each accession are shown in Table 2. Among diploid species, the most common karyotype formula was 8m+6sm for *F. bucharica* Losinsk. PI 551851, *F.*×*bifera* Duchesne PI 616613, *F. chinensis* PI 551576 and *F. nilgerrensis* Schltld. ex J. Gay PI 637950, and followed in frequency of 10m+4sm for *F. mandshurica* CFRA 1947, *F. vesca* subsp. *americana* (Porter) Staudt PI 552287, *F. vesca* subsp. *bracteata* (A. Heller) Staudt PI 637947 and *F. vesca* subsp. *californica* (Cham. and Schltld.) Staudt PI 551513. On the other hand, *F. vesca* subsp. *vesca* PI 551826 and *F. vesca* subsp. *vesca* f. *roseiflora* (Boulay) Staudt PI 616872 had a karyotype formula as 8m+4sm+2st, and *F. pentaphylla* PI 651570 had 10m+2sm+2st. The karyotype features of the tetraploid species accessions presented only m and sm chromosomes. *F. tibetica* Staudt & Dickoré PI 651567 had the highest number of m chromosomes among the tetraploids. The karyotype formula of *F. moschata* PI 551550 was 30m+10sm+2st (Table 2). In addition, except for *F. orientalis* Losinsk. PI 602942, in most karyotypes, the longest pairs of chromosome complements were the m chromosome (Fig. 4).

The range of chromosome lengths was 0.61–0.93 μm in *F. chinensis* PI 651576 and 0.93–1.85 μm in *F. orientalis* PI 602942 (Table 2). The TCL value varied significantly from 11.00 μm in *F. chinensis* PI 551576 to 43.54 μm in *F. moschata* PI 551550. The highest value of

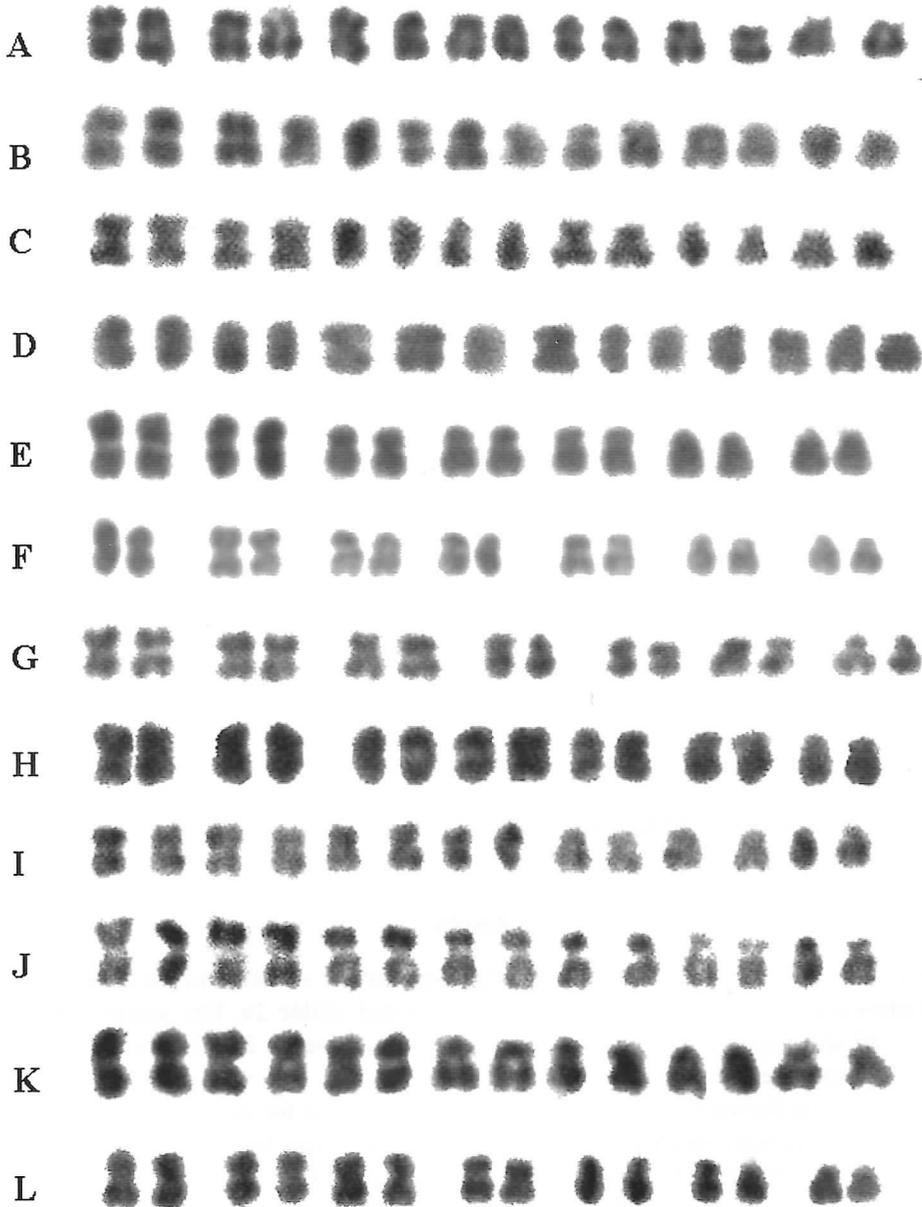


Fig. 3. The karyotypes of wild diploid strawberries species. (A) *F. × bifera* PI 616613. (B) *F. bucharica* PI 551851. (C) *F. chinensis* CFRA202. (D) *F. mandshurica* CFRA 1947. (E) *F. nilgerrensis* PI 637950. (F) *F. pentaphylla* PI 651570. (G) *F. vesca* subsp. *americana* PI 552287. (H) *F. vesca* subsp. *bracteata* PI 637947. (I) *F. vesca* subsp. *californica* PI 551513. (J) *F. vesca* subsp. *vesca* PI 551826. (K) *F. vesca* subsp. *roseiflora* PI 616872. (L) *F. viridis* PI 616609.

TCL ($43.54 \mu\text{m}$) was observed in *F. moschata* PI 551550. The longest and shortest MCL was $1.31 \mu\text{m}$ for *F. nilgerrensis* PI 637950 and $0.79 \mu\text{m}$ for *F. chinensis* PI 616699. There were no significant differences in the CI values for all accessions. The highest CI value of 39.50 was observed in *F. vesca* subsp. *americana* PI 552287. *F. chinensis* PI 551576 and *F. orientalis* Losinsk. PI 602942 exhibited lower CI values than other species.

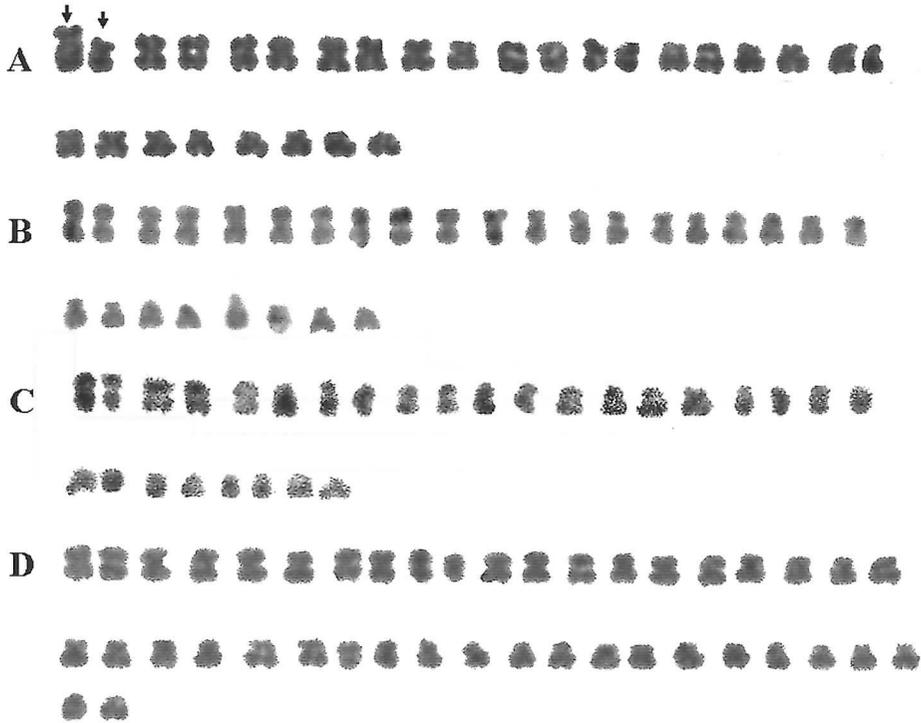


Fig. 4. The karyotype of tetraploid strawberries ($2n=28$), *F. orientalis* PI 602942 (A), *F. corymbosa* CFRA 1911. (B), *F. tibetica* PI 651567 (C), and hexaploid strawberry ($2n=42$), *F. moschata* PI 551550 (D). Arrows indicate the largest sm chromosome pairs, which might be the pair of sex chromosomes.

A dendrogram constructed based on the numerically karyotype data showed two major clades (Fig. 5). The first clade included all of the diploid species, and one tetraploid accession, *F. tibetica* PI 651567. Within this major cluster the wild Eurasia diploid species were classified as members of the same sub-subclade, with the exception of *F. pentaphylla* PI 651570, and differ from the accessions of *F. vesca* subspecies from America and Europe. The second major clade was comprised of the wild tetraploid *F. corymbosa* CFRA 1911, *F. orientalis* PI 551864, *F. orientalis* PI 602942, and hexaploid *F. moschata* PI 551550 accessions.

Discussion

The results of chromosome counting confirmed that the somatic chromosomes of wild diploid, tetraploid and hexaploid strawberry species were $2n=2x=14$, $2n=4x=28$ and $2n=6x=42$, respectively (Table 2). These results were consistent with descriptions in previous reports (Darrow 1966, Ichijima 1926, Iwatsubo and Naruhashi 1989, 1991, Lilienfeld 1933, Lim 2000, Naruhashi *et al.* 1999, Nathewet *et al.* 2007, Staudt 1999, 2003, 2005, 2006, Staudt and Dickoré 2001, Staudt *et al.* 2003, Yarnell 1929). This study is also the first report of some previously unreported karyotypes for diploid (*F. bucharica*, *F. × bifera*, *F. chinensis*, *F. nilgerrensis*, *F. pentaphylla*, *F. viridis* and subspecies of *F. vesca*), tetraploid (*F. orientalis*, *F. corymbosa* and *F. tibetica*) and hexaploid (*F. moschata*). The results of karyotype analysis for the diploid species in this study are similar with the results for diploid *Fragaria* (Iwatsubo and Naruhashi 1989, 1991).

Our results also confirmed that the chromosome lengths of wild strawberry plants in genus

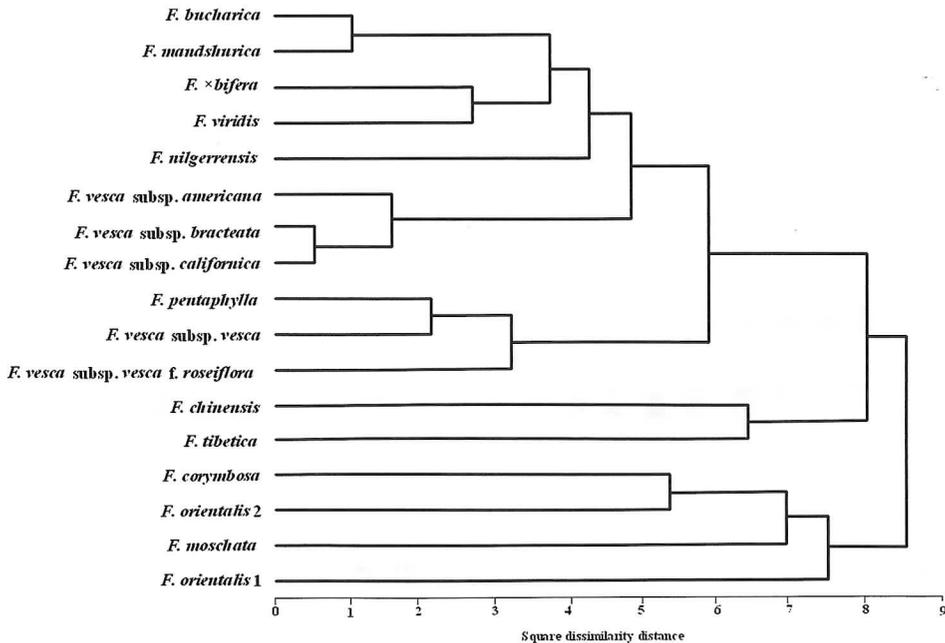


Fig. 5. Dendrogram demonstrating phenetic relationships among the diploid, tetraploid and hexaploid species in *Fragaria*.

Fragaria were small (Iwatsubo and Naruhashi 1989, 1991, Nathewet *et al.* 2007, Naruhashi *et al.* 1999, Yamaguchi 1980). The mean chromosome length for the diploid, tetraploid and hexaploid species was $1.09 \mu\text{m}$. The total chromosome lengths in each species increased according to their ploidy level. The highest value of TCL ($43.54 \mu\text{m}$) was recorded in *F. moschata*, a hexaploid. In addition, the present study suggested that each diploid species had a greater uniformity in karyotype formulae and similarity of quantitative data than the tetraploid species. Particularly, the karyotype formulae, TCL and CI values among the Asian wild diploid species were similar and consistent. This finding is consistent with that of Iwatsubo and Naruhashi (1989, 1991), who reported that of 5 diploid *Fragaria*, *F. daltoniana*, *F. nipponica*, *F. nubicola*, and *F. vesca* displayed similar karyotype morphology to each other, while *F. iinumae* was significantly distinctly related to the other diploids. In addition, our results have shown that the tetraploid species have a similar karyotype formula but their TCL differs, suggesting that the addition of genetic material may also be involved in their karyotype evolution. Although, in this study, the karyotypic differences that we observed among the diploids and tetraploids were small, the species could be differentiated by the number and type of chromosomes, and statistical differences of the means of numerical data such as TCL, MCL and CI values.

Furthermore, in most karyotypes, the longest pairs of complements were m chromosomes. Surprisingly, the longest pair of chromosomes in *F. orientalis* PI 602942 was the largest sm chromosome pair. The largest sm chromosomes of PI 602942 in *F. orientalis* were confirmed using more than 10 root tips, and those chromosomes exist in almost all somatic cells. These chromosomes might be sex chromosomes of *F. orientalis* PI 602942 (Fig. 4A) because the plant in this species consists of dioecious and trioecious populations (Staudt 2003). The existence of sex chromosomes had been reported in the pollen mother cells of hexaploid *F. moschata* (Kihara, 1930). Recently, Nathewet *et al.* (2009) reported sex chromosomes in the mitotic cells of staminate male *F. chiloensis* plants. In this study, however, we were not able to confirm the identity of the sex chromosomes in mitotic cells of *F. moschata*.

Karyotype similarities were evaluated using cluster analysis to clarify phylogenetic relations among or within the species of diploid, tetraploid, and hexaploid species (Fig. 5). The wild diploid and tetraploid species, with the exception of the tetraploid, *F. tibetica* PI 651567, could be clearly separated using gross karyotype morphology. These species can also be separated based on morphological, cytological, ecological characteristics and molecular marker analysis (Hadonou *et al.* 2004, Potter *et al.* 2000, Staudt 1962, 1989, 1999, 2003, 2006, Staudt and Dickoré 2001, Staudt *et al.* 2003). Our cluster analysis suggested that most wild Eurasian diploid species accessions clustered together, but the American and European *F. vesca* subspecies were distinct. Sargent *et al.* (2004) and Potter *et al.* (2000) reported distinct morphological and molecular biology aspects, respectively, between *F. vesca* accessions and other diploids. This is reflected by the geographical distribution and genetic and morphological distinctness of these species. Within the group of wild Eurasian diploid species, *F. × bifera* clustered with *F. viridis*, which was to be expected, as *F. viridis* is known to be a parental *F. × bifera* (Staudt *et al.* 2003). Furthermore, the American subspecies of *F. vesca* (*F. vesca* subsp. *americana*, *F. vesca* subsp. *bracteata* and *F. vesca* subsp. *californica*) were clearly separated from the *F. vesca* subsp. *vesca* from Europe (Fig. 5), reaffirming the long genetic divergence of the American and European *F. vesca* subspecies. These different relationships were consistent with results of non-coding nuclear sequences analyses (Potter *et al.* 2000). Hadonou *et al.* (2004) also reported high similarity of molecular biological aspects among American *F. vesca* subspecies. For tetraploid species, the analysis of phylogenetic relationships based on molecular and biological aspects showed that all tetraploid species were clustered in the same group (Potter *et al.* 2000, Rousseau-Gueutin *et al.* 2009). In contrast, our results showed that *F. tibetica* PI 651567 was grouped with the diploid species, *F. chinensis* and separated from the tetraploid group. Moreover, *F. moschata* clustered in the same clade with the tetraploid species, *F. orientalis* and *F. corymbosa*, indicating that *F. moschata* is closely related to the tetraploid species. This relationship was supported by Harrison *et al.* (1997) and Potter *et al.* (2000) who claimed that these 2 species are indeed sister taxa. However, there are some questions about morphological classification of *F. orientalis* PI 551864, which was also used in a previous study (Harrison *et al.* 1997, Potter *et al.* 2000). This particular accession presumably derived from open pollination and may not be pure *F. orientalis*, so the NCGR is now classified as a hybrid. Moreover, the parents of *F. orientalis* PI 551864 are still unknown. If the maternal plants were correctly labeled, then our results may accurately represent the *F. orientalis* karyotype.

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