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Chapter 2 *Fragaria*

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Chapter 2
Fragaria

Kim E. Hummer, Nahla Bassil, and Wambui Njuguna

2.1 Botany

2.1.1 Taxonomy and Agricultural Status

Strawberry, genus *Fragaria* L., is a member of the family Rosaceae, subfamily Rosoideae (Potter et al. 2007), and has the genus *Potentilla* as a close relative. Strawberry fruits are sufficiently economically important throughout the world such that the species is included in The International Treaty on Plant Genetic Resources, Annex 1 (*http://www.planttreaty.org/*).

The hybrid strawberry fruit of commerce, *Fragaria×ananassa Duchesne ex Rozier* nothosubsp. *ananassa*, is eaten by millions of people and is cultivated from the arctic to the tropics. More than 75 countries produce significant amounts of this fruit (FAO 2010). Annual world production is increasing from 3 to more than 4 thousand MT (Fig. 2.1). About 98% of the production occurs in the Northern Hemisphere, though production is expanding in the south (Hummer and Hancock 2009).

The genus *Fragaria* was first summarized in pre-Linnaean literature by C. Bauhin (1623). In *Hortus Cliffortianus*, Linneaus (1738) described this genus as monotypic containing *Fragaria flagellis reptan*; in *Species Plantarum* (Linneaus 1753), he described three species including varieties, though several European species now known were omitted, and one belonging to *Potentilla* was included (Staudt 1962).

Duchesne (1766) was credited for publishing the best early taxonomic treatment of strawberries (Hedrick 1919; Staudt 1962). Duchesne maintained the strawberry collection at the Royal Botanical Garden, having living collections documented from various regions and countries of Europe and the Americas. He distributed samples to Linnaeus in Sweden.

The present *Fragaria* taxonomy includes 20 named wild species, three described naturally occurring hybrid species, and two cultivated hybrid species important to commerce (Table 2.1). The wild species are distributed in the north temperate and holarctic zones (Staudt 1989, 1999a, b; Rousseau-Gueutin et al. 2008). European and American *Fragaria* subspecies were monographed by Staudt (1999a, b), who also revised the Asian species (Staudt 1999a, b, 2003, 2005; Staudt and Dickorè 2001). Chinese and mid-Asian species are under study (Lei et al. 2005) but require further collection and comparison, considering global taxonomy. The distribution of specific ploidy levels within certain continents has been used to infer the history and evolution of these species (Staudt 1999a, b).

2.1.2 Geographical Locations of Species

Fragaria species exist as a natural polyploid series from diploid through decaploid (Table 2.1). Diploid *Fragaria* species are endemic to boreal Eurasia and North America. *Fragaria vesca* is native from the west of the Urals throughout northern Europe and across the North American continent. However, this diploid species is not native to Siberia, Sakhalin, Hokkaido, Japan, Kamchatka, or to the Kurile, Aleutian, or Hawaiian Archipeliagos according to flora of those regions (Hultén 1968). It has been introduced in many of those areas.

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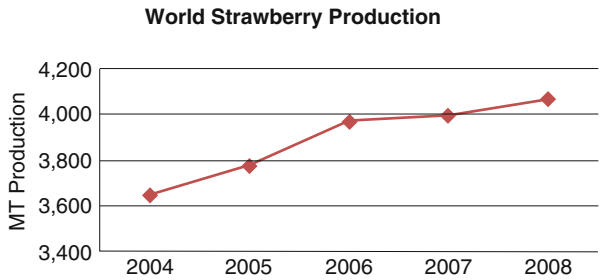

Fig. 2.1 World Strawberry production

Table 2.1 *Fragaria* species, ploidy, and distribution area

F. bucharica Losinsk	2*x*	Western Himalayas
F. chinensis Losinsk[a]		China
F. daltoniana J. Gay		Himalayas
F. iinumae Makino		Japan
F. mandshurica Staudt		North China
F. nilgerrensis Schlect.		Southeastern Asia
F. nipponica Makino		Japan
F. nubicola Lindl.		Himalayas
F. pentaphylla Losinsk		North China
F. vesca L.		Europe, Asia west of the Urals, disjunct in North America
F. viridis Duch.		Europe and Asia
F. ×*bifera* Duch.		France, Germany
F. corymbosa Losinsk	4*x*	Russian Far East/China
F. gracilis A. Los.		Northwestern China
F. moupinensis (French.) Card		Northern China
F. orientalis Losinsk		Russian Far East
F. tibetica Staudt & Dickoré		China
F. ×*bringhurstii* Staudt	5*x* (9*x*)	California
F. sp. nov[b]		China
F. moschata Duch.	6*x*	Euro-Siberia
F. chiloensis (L.) Miller	8*x*	Western N. America, Hawaii, Chile
F. virginiana Miller		North America
F. ×*ananassa* Duch. ex Lamarck		Cultivated worldwide
F. ×*ananassa* subsp. *cuneifolia*		Northwestern N. America
F. iturupensis Staudt	10*x*	Iturup Island, Kurile Island
F. virginiana subsp. platypetala Miller		Oregon, United States
F. ×*vescana* R. Bauer & A. Bauer		Cultivated in Europe

[a]As proposed by Staudt (2008)
[b]As proposed by Lei et al. (2005)

Diploid strawberry species are reported on many of the islands of and surrounding Japan, in Hokkaido, on Sakhalin, and in the greater and lesser Kuriles (Makino 1940).

Diploid and tetraploid species are native not only to Asia, particularly in China, but also in Siberia and the Russian Far East. Wild, naturally occurring pentaploids ($2n = 5x = 35$) have been observed in California (*F.* ×*bringhurstii*) and China (Lei et al. 2005). These strawberries exist in colonies with other ploidy levels nearby. The only known wild hexaploid ($2n = 6x = 42$) species, *F. moschata*, is native to Europe as far east as Lake Baikal. This species is commonly known as the musk strawberry (Hancock 1999).

Wild octoploid species are distributed from Unalaska eastward in the Aleutian Islands (Hultén 1968),

completely across the North American continent, on the Hawaiian Islands, and in South America (Chile) (Staudt 1999a, b). Wild decaploids are native to the Kurile Islands (*F. iturupensis*) (Hummer et al. 2009) and the old Cascades in western North America (Hummer unpublished).

2.1.3 Description of Wild Species Relatives

2.1.3.1 Diploids

Fragaria vesca, a self-compatible, sympodial-runnering diploid (Staudt et al. 2003), has the largest native range (presently) among *Fragaria* species. It is the only diploid species with disjunct subspecies in North America. *Fragaria vesca* has four subspecies: the European *F. vesca* subsp. *vesca*, the American *F. vesca* subsp. *americana*, *Fragaria vesca* subsp. *bracteata*, and *F. vesca* subsp. *californica*. *Fragaria vesca* subsp. *vesca* is endemic across Europe eastward to Lake Baikal (Staudt 1989). Several forms of *Fragaria vesca* subsp. *vesca* species have been identified but more common ones include forma *vesca*, f. *semperflorens* and f. *alba*. *Fragaria vesca* subsp. *americana* is distributed in many US states from Virginia, to South Dakota, North Dakota, Missouri, Nebraska, and Wyoming. This subspecies is also found in Ontario, Canada, and British Columbia. *Fragaria vesca* subsp. *americana* differs from other subspecies by its slender morphological structure. *Fragaria vesca* subsp. *bracteata* occurs around the coastal and Cascade mountain ranges from British Columbia through Washington and Oregon, and the Sierra Nevada in California. Its distribution extends into Mexico where it is referred to as *F. mexicana* Schltdl. (Staudt 1999b). This chapter uses Staudt's (1999b) treatment where *F. mexicana* is submerged under *F. vesca* subsp. *bracteata*.

While the other three *vesca* subspecies are hermaphroditic, some genotypes of *F. vesca* subsp. *bracteata* are reported as gynodioecious (Staudt 1989). *Fragaria vesca* subsp. *californica* occurs near the Pacific Ocean from southern Oregon to California. Hybrids of *F. vesca* subsp. *californica* and subsp. *bracteata* have been observed in regions of overlap where subsp. *bracteta* approaches the coastal range distribution of subsp. *californica*.

In Europe, *F. vesca* subsp. *vesca* overlaps in distribution with another diploid, *F. viridis*, which has a monopodial branching system of the runners, a feature used to distinguish the two species. The fruit of *F. viridis* has wine red skin while the cortex and pith is yellowish–greenish and the fruit does not easily detach from the calyx (Staudt et al. 2003). In regions where *F. vesca* and *F. viridis* distributions overlap including Russia, Germany, France, Finland, and Italy, hybridization has occurred resulting in the hybrid species *F.* ×*bifera*. Morphological features of this hybrid species are mostly intermediate and include the stolon branching system and leaf color. The fruit, like *F. viridis*, does not easily detach from the calyx. In addition, the fruit has pigment only in the skin as is the case with *F. viridis*, and the fruits are embedded in shallow pits, a feature found in *F. vesca*. The triploid form of the hybrid that includes two genome copies from *F. vesca* seems to be similar to *F. vesca* in certain features such as the easy detachment of fruit from the calyx, flesh texture, smell, and taste of the fruit (Staudt et al. 2003).

Fragaria mandschurica has sympodially branched runners and hermaphrodite flowers with functional stamens and fruit that shows good seed set. This diploid is distributed on the east banks of Lake Baikal and is also found in Mongolia and South Korea and spreads to northeastern China. The tetraploid *F. orientalis* overlaps in distribution with *F. mandshurica* in the Amur Valley of China and is also distributed in Russia.

Fragaria nilgerrensis is a self-compatible diploid with two subspecies: subsp. *nilgerrensis* and subsp. *hayatae* Makino (Staudt 1999a). The fruit of *F. nilgerrensis* subsp. *nilgerrensis* is white to cream and is distributed in northwestern and southwestern India, East Himalaya, northeastern Burma, northern Vietnam, Southwest and central China. Despite this wide distribution of the subspecies, only limited morphological variation has been observed among different populations. The fruit of *F. nilgerrensis* subsp. *hayatae* has pink to red skin, a cream colored cortex (Staudt 1999a), and is known for its high anthocyanin levels in all plant parts including the berries (Staudt 1989). In contrast to the wide distribution of *F. nilgerrensis* subsp. *nilgerrensis*, subsp. *hayatae* is only recorded in Taiwan. The leaf morphology of the tetraploid *F. moupinensis*, distributed in Yunnan and Sichuan provinces of China and in Tibet, resembles that of *F. nilgerrensis* (Darrow 1966).

Fragaria daltoniana J. Gay is a self-compatible diploid with sympodial runners with elongate conical white to pinkish fruit. Hybridization with other diploids has been previously tested, but the results were not published and were only stated in Staudt (2006). Hybrids with *F. iinumae*, *F. nilgerrensis*, and *F. nipponica* Makino were morphologically intermediate. The diploid *F. daltoniana* is distributed in the Himalayas from India to Myanmar (Staudt 2006).

Like *F. daltoniana*, the diploid *F. bucharica* is found in the Himalayan region but is self-incompatible. It has sympodial runners, a characteristic that distinguishes it from *F. nubicola* (Hook. f.) Lindl. ex Lacaita also found in the Himalayas. Two subspecies of *F. bucharica*, subsp. *bucharica* and subsp. *darvasica*, are recognized and are currently only distinguished by the size of bractlets: they are smaller in subsp. *darvasica* than in subsp. *bucharica*. Crossability tests with other diploids including *F. mandshurica*, *F. vesca*, and *F. viridis* resulted in mostly heterotic plants with *F. bucharica* morphological characters prevailing, even with reciprocal crosses. In contrast, crosses with *F. nipponica* produced dwarf plants. *Fragaria bucharica* is distributed from Tadjikistan to Afghanistan, Pakistan, and Himachal Pradesh in India (Staudt 2006). Another diploid species frequently confused with *F. bucharica* due to the similar morphological characteristics and also found in the Himalayas is *F. nubicola*. This diploid is self-incompatible with a monopodial branching pattern of the stolon, which is the only distinguishing feature separating it from *F. bucharica*. It is distributed along the southern slopes of the Himalayas to Southeast Tibet, and in Southwest China. *Fragaria nubicola* was observed to form accessory leaflets probably associated with the time of year.

Fragaria pentaphylla is a self-incompatible diploid found in China. *Fragaria pentaphylla* f. *alba* Staudt and Dickoré, only known from Mt. Gyala Oeri and north of the Tsangpo Gorge in Tibet, has only been identified from a white-fruited population. Red-fruited types are expected with further exploration of this region (Staudt and Dickorè 2001). As the name "pentaphylla" suggests, this species contains accessory leaflets. However, the presence of accessory leaflets is not restricted to this species but has been seen in other strawberry species throughout the world, including *F. nubicola* and the tetraploid, *F. tibetica*. The formation of accessory leaflets has been associated with certain times of the year as noted by Staudt and Dickorè (2001). Strawberry plants show accessory leaflets to be a common characteristic in many species including *F. virginiana*, *F. chiloensis*, and *F. iturupensis*. *Fragaria pentaphylla* is closely related to a tetraploid species, *F. tibetica*, which also has a white-fruited form, *F. tibetica* f. *alba*. The two species are distinguished from each other by the heteroecy, tetraploidy, larger pollen grains, and larger achenes found in *F. tibetica*. The distribution of the tetraploid extends from Central and Eastern Himalaya to the Chinese provinces, Yunnan and Sichuan. *F. pentaphylla* and *F. tibetica* have monopodial runners and can therefore be distinguished from Himalayan *F. nubicola* and *F. daltoniana* that have sympodial runners. *Fragaria iinumae* is found in the lowlands of Hokkaido in the north to the mountains of the main island Honshu in areas of heavy snow along the Sea of Japan (Hancock 1999). *Fragaria iinumae* is known for its unique characters not found in other *Fragaria* diploids such as the glaucous leaves. It has sympodial runners and its flowers have six to nine petals per flower, while *Fragaria* flowers commonly have five. Due to its glaucous leaves, this diploid may be a progenitor of the octoploid species, *F. virginiana* (Staudt 2005). The crowns of *F. iinumae* usually appear as rosettes, but they sometimes rise above the ground in "tufts" making this species conspicuous (Oda 2002).

Fragaria nipponica, a diploid, which now includes the submerged species *F. yezoensis* (Naruhashi and Iwata 1988), is a self-incompatible species distributed in Honshu and Hokkaido in Japan, and, Sakhalin and Kuriles in Russia (*F. nipponica* subsp. *nipponica*), Yakushima Islands of Japan (*F. nipponica* subsp. *yakusimensis*), and in the Island of Cheju-do off the Korean mainland (*F. nipponica* subsp. *chejuensis*) (Staudt 2008). Tetraploid hybrids of *F. nipponica* subsp. *nipponica* with *F. moschata* (*F. nipponica* as the maternal parent) provided evidence of homology of the *F. moschata* and *F. nipponica* genomes (Staudt 2008). *F. iinumae* and *F. nipponica* are the only diploid species endemic to Japan and the islands north of Japan including the Kuriles. *F. nipponica* is confined to the Pacific Ocean side of Japan while *F. iinumae* is found on the Sea of Japan side (Staudt 2005). During the winter, aboveground shoots of *F. iinumae* die back, though the crown and roots remain alive.

2.1.3.2 Tetraploids

Known named tetraploid species occur in Southeast and East Asia. Staudt (2006) proposed that four tetraploid species may have originated as the first step of ploidization from diploid species.

The diploid *F. pentaphylla* seems to be the putative ancestor of the tetraploid *F. tibetica*, given their distribution and similar morphological characteristics (Staudt and Dickorè 2001). Two tetraploid species, *F. corymbosa* and *F. moupinensis*, may have been derived from the diploid *F. chinensis* (Staudt 2003).

Similarity in morphological characters of *F. mandschurica* and *F. orientalis* and their sympatry in far eastern Russia was proposed to support *F. mandshurica* as the diploid ancestor of the tetraploid *F. orientalis* (Staudt 2003). The tetraploid *F. orientalis* can be distinguished from *F. mandshurica* by the size of its pollen grains, a characteristic related to the number of chromosomes. Though *F. mandshurica* is hermaphroditic, *F. orientalis* contains both dioecious and trioecious populations.

2.1.3.3 Hexaploid

The sole hexaploid species, *F. moschata*, grows in forests, under shrubs and in tall grass (Hancock 1999). Like the diploids *F. vesca* and *F. viridis*, *F. moschata* is native to northern and central Europe. This species was extensively cultivated in Europe (France and Germany) from 1,400 to 1,850 due to its desirable flavor and aroma. The fruit only has color on the skin, while the cortex and pith are yellowish-white, with a strong, musky smell and taste (Staudt et al. 2003). The populations are dioecious (Staudt et al. 2003), which contributes to scanty yields in comparison to cultivated hermaphroditic diploid and octoploid species (Hancock 1999). *Fragaria vesca*, *F. viridis*, and *F. moschata* are sympatric with *F. mandshurica* to the east (Staudt 2003).

2.1.3.4 Octoploids

Fragaria chiloensis, known as the beach strawberry, is an American octoploid. This species is divided into four subspecies. The two northerly distributed subspecies are *F. chiloensis* subsp. *pacifica* and *F. chiloensis* subsp. *lucida*. These subspecies are found along sandy beaches of the Pacific Ocean from Alaska to California and have small red fruit.

Fragaria chiloensis subsp. *sandwicensis* is distributed in mountainous regions of Hawaii and Maui (Staudt 1999b).

Fragaria chiloensis subsp. *chiloensis* f. *patagonica*, also red-fruited, is distributed in coastal mountains, the central valley in Chile, and in the Andes in southern Chile with the southern limit of its distribution in Argentina. *Fragaria chiloensis* subsp. *chiloensis* f. *chiloensis* is cultivated in Chile, Ecuador, and Peru. White-fruited landrace of *F. chiloensis* was first domesticated by the Mapuche Indians. This forma has larger flower and fruit structures than other *F. chiloensis* subspecies. This large, white-fruited landrace with hairy petioles was imported from Chile to Europe in the early eighteenth century. It is the maternal progenitor of the cultivated strawberry (Darrow 1966; Hancock 1999).

Fragaria virginiana is native to North America. This species is also known as the "scarlet" strawberry. *Fragaria virginiana* subsp. *virginiana* is the paternal progenitor of the cultivated strawberry (Hancock 1999).

Wild *F. virginiana* is divided into four subspecies. *Fragaria virginiana* subsp. *virginiana* is found throughout eastern North America and spreads to British Columbia in the west (Harrison et al. 2000). *Fragaria virginiana* subsp. *grayana* (Vilm. ex J. Gay) Staudt is found from northwestern Texas, to Nebraska, Iowa, and Illinois. It is also found in Louisiana, Alabama, Indiana, Ohio, Virginia, and New York.

The distribution of *F. virginiana* subsp. *glauca* resembles that of subsp. *virginiana*; however, this species spreads further west in British Columbia interacting with *F. chiloensis* found along the coast (Staudt 1999b). *Fragaria virginiana* subsp. *glauca* is distinguished from other subspecies by the smooth leaf surface and the dark to light bluish (glaucous) leaves.

The leaves of *F. virginiana* subsp. *platypetala* are also blue green but only slightly (Staudt 1999b). *Fragaria virginiana* subsp. *platypetala* is distributed in British Columbia and extends southward to Washington, Oregon, and northern California (Staudt 1999b). Further south in British Columbia, *F. virginiana* subsp. *glauca* overlaps in distribution with subsp. *platypetala* (Rydb.) Staudt, and introgression has been encountered.

Fragaria ×*ananassa* subsp. *cuneifolia* is suspected as a natural hybrid of *F. chiloensis* subsp. *pacifica* or

subsp. *lucida* and *F. virginiana* subsp. *platypetala* (Staudt 1999b). Unlike the cultivated strawberry of commerce, this hybrid has smaller leaves, flowers, and fruits. The distribution of *F.* ×*ananassa* subsp. *cuneifolia* is from the coastal regions of British Columbia (Vancouver Island) south to Fort Bragg and Point Arena lighthouse in California. Hybrids of *F.* ×*ananassa* subsp. *cuneifolia* and the two octoploids, *F. chiloensis* subsp. *pacifica* and *F. virginiana* subsp. *platypetala*, have been seen in Oregon, Washington, and California in the US (Staudt 1999b).

2.1.3.5 Decaploids

Fragaria iturupensis is a polyploid strawberry distributed on the eastern slopes of Mt. Atsonupuri on Iturup, the second island in the southern section of the greater Kuril Island archipelago. This species has a limited distribution of a few colonies on the rock skree on the eastern flank of the volcano. This location might have provided a refugium from the most recent glaciations, which is reported to have come only as far south as the northern part of Iturup Island.

In 1973, chromosome counts of *F. iturupensis* indicated that this species was octoploid (Staudt 1989). Those initial plants were lost. A return trip to Atsonupuri in 2003 obtained another sample of *F. iturupensis*. Chromosome counts and flow cytometry indicated this sample to be decaploid. (Hummer et al. 2009). *Fragaria iturupensis* resembles *F. virginiana* subsp. *glauca* (Staudt 1989) and *F. iinumae* (Hancock 1999) in leaf texture and color. The oblate fruit shape and erect inflorescence and flavor components of this polyploid population resemble those found in *F. vesca* (Staudt 2008).

Staudt (1999a, b) postulated that *F. iturupensis* is more primitive than *F. virginiana* subsp. *glauca*. Thus far, molecular analyses have concurred (Njuguna et al. 2010).

2.1.3.6 Unusual Ploidy

Fragaria ×*bringhurstii* is a hybrid species between *F. chiloensis* and *F. vesca* subsp. *californica*. This species is distributed near the Pacific Ocean in California in Humboldt and Monterey counties (Staudt 1999b). Varying levels of morphological intermediacy between *F. chiloensis* and *F. vesca* were observed in the hybrid species. Genotypes of this species with different ploidy levels including pentaploid ($2n = 5x = 35$), hexaploid ($2n = 6x = 42$), and enneaploid ($2n = 9x = 63$) have been found.

In 2009, plants were morphologically similar to *F. virginiana* subsp. *platypetala* but appeared decaploid based on microsatellite analysis and flow Cytometry (Wambui Njuguna and Nahla Bassil unpublished). Nathewet et al. (2009) confirmed decaploidy by chromosome counts. These plants occurred in the Oregon Cascades near the Pacific Crest Trail where it is conspecific with *F. vesca* subsp. *bracteata*. The occurrence of multiple ploidy levels in *F. virginiana* subsp. *platypetala* is suspect where its distribution overlaps with *F. vesca* subspecies.

2.1.4 Strawberry History of Cultivation

E. L. Sturtevant, through U. P. Hedrick (1919) and Darrow (1966), describes early references for European strawberry from the Ancient Roman verses of Virgil and Ovid and the glancing mention in Pliny's *Natural History*. Darrow (1966) pointed out that this fruit was not a "staple of agriculture" to explain its exclusion from Theophrastus, Hippocrates, Dioscorides, or Galen.

By the 1300s, the French began transplanting *F. vesca*, the wood strawberry, from the wilderness into the garden. In 1368, King Charles V had his gardener, Jean Dudoy, plant no less than 1,200 strawberries in the royal gardens of the Louvre, in Paris (Darrow 1966). Written references to the strawberry in Shakespeare and his contemporaries may indicate the success of the plant in the gardens of that time. In 1530, King Henry VIII paid ten shillings for a "pottle of strawberries" (slightly less than 250 g) according to his Privy Purse Expenses (Darrow 1966).

In addition to the alpine strawberry, Darrow (1966) noted that *F. moschata* was cultivated in Europe. Karp (2006) described this species as the most aromatic strawberry. *F. viridis*, the "green" strawberry, was also gathered and eaten.

Between the tenth and the eighteenth centuries, in Japan, the ancient word "ichibigo" referred to many

berry crops (including Japanese strawberry species and the low-growing *Rubus pseudo-japonica*) gathered from the wild (Oda and Nishimura 2009). The word migrated to "ichigo", now the term of reference for the modern day *Fragaria* species. The cultivated *F.* ×*ananassa* was first brought into Japan from the Netherlands in the early to mid-nineteenth century.

The Virginia strawberries impacted the European strawberry industry of the 1800s with their high yields and deep red color, resulting in the name "scarlet strawberry". The scarlet strawberry was cultivated in Europe, and some important cultivars included: "Oblong Scarlet", "Grove End Scarlet", "Duke of Kent's Scarlet", and "Knight's Large Scarlet".

At the time of the reintroduction of the scarlet strawberry to the United States in the early 1700s, *F. virginiana* plantings were established in Boston, New York, Philadelphia, and Baltimore. "Hudson", a vigorous, soft-fruited and high flavored *F. virginiana* clone, was considered the first most important American strawberry (Hancock 1999). The attractive color, large size and acceptable flavor made it favorable for making jam. It was used through the early part of the twentieth century (Fletcher 1917). Desirable horticultural traits, such as winter hardiness, frost tolerance, resistance to red stele, adaptation to diverse environmental conditions, and interfertility with the cultivated strawberry (Hancock et al. 2002), made *F. virginiana* a valuable genetic resource for breeders. A *F. virginiana* subsp. *glauca* clone from Hecker Pass was the primary source of the day-neutral trait in the cultivar development program of California in the 1970s and 1980s.

Importation of Chilean clones to Europe in the early eighteenth century resulted in the accidental hybridization with *F. virginiana* subsp. *virginiana* from North America, forming the now cultivated *F.* ×*ananassa* subsp. *ananassa*, now known as the hybrid of commerce. *Fragaria chiloensis* has been used in breeding programs as a source of winter hardiness (Staudt 1999b), resistance to strawberry root disease, and virus tolerance (Lawrence et al. 1990).

Fragaria ×*ananassa*, the "pineapple strawberry", was the species name given to the accidental hybrid of *F. chiloensis* subsp. *chiloensis* f. *chiloensis* and *F. virginiana* subsp. *virginiana* in Europe by Duschesne in the early eighteenth century (Hancock 1999).

Since the mid-1800s, breeding in Europe and United States has resulted in hundreds of cultivars from 35 breeding programs (Faedi et al. 2002). The *F.* ×*ananassa* subsp. *ananassa* includes these cultivated species originating from the accidental hybrids first recognized in France around 1750. Breeding work in Alaska utilized *F. chiloensis* to develop Sitka hybrids that were winter hardy and suited for climatic conditions in Alaska (Staudt 1999b).

In North America, natural hybridization between *F.* ×*ananassa* subsp. *ananassa*, which escapes cultivation, with subspecies of *F. chiloensis* and *F. virginiana* have been observed. These hybrids are usually identified in the wild by the large berries, sometimes erratic fruit set, and fruit taste. *Fragaria chiloensis* populations resulting from introgression into the hybrid octoploid were observed in California (*F. chiloensis* subsp. *lucida*) and Chile (*F. chiloensis* subsp. *chiloensis* f. *patagonica*). Introgression of the cultivated strawberry into wild populations of *F. virginiana* subsp. *grayana* occurs in the southeastern United States.

2.1.5 Tribal Use of Primitive Forms

In South America, the Mapuche (Mäpfuchieu) and Huilliche Indians, the indigenous people of central and southern Chile, cultivated strawberries. Their economy was based on agriculture until the appearance of the Spanish conquistadores. They developed a landrace of the white Chilean strawberry (*F. chiloensis* subsp. *chiloensis* f. *chiloensis*) and cultivated this fruit, undisturbed for thousands of years until 1550–1551.

The Spanish considered this fruit as a spoil of conquest. Pedro de Valdivia and his men brought this fruit to Cuzco, Peru, in 1557, where it was described as the "chili" (Darrow 1966).

Spread of the Chilean berries to other countries within South America followed the Spanish invasion (Hancock 1999).

Strawberry acreage found in Ecuador was reported to be largest observed in South America during the period between 1700 and 1970 (Finn et al. 1998). Despite the higher yields obtained with *F.* ×*ananassa* in Chile (20–70 t/ha), its flavor and aroma have been described as lower than that of *F. chiloensis* (Retamales et al. 2005). High-yielding *F.* ×*ananassa* cultivars displaced the local Chilean landrace cultivars in the twentieth century (Retamales et al. 2005).

2.2 Phylogeny

In *Fragaria*, phylogenetic analysis has been attempted using chloroplast and nuclear genome sequences, but most species relationships have remained unclear. Harrison et al. (1997b) used restriction fragment length variation of chloroplast DNA from nine species, while Potter et al. (2000) used the nuclear internal transcribed spacer (nrITS) region and the chloroplast *trn*L intron and the *trn*L–*trn*F spacer region in 14 species. Low resolution of the phylogenetic tree from these two studies was speculated to be due to little divergence of the genome regions investigated (Rousseau-Gueutin et al. 2009).

The *Fragaria* octoploid genome models AAA′A′BBB′B′ (Bringhurst 1990), and the more recently published YYY′Y′ZZZZ/YYYYZZZZ models (Rousseau-Gueutin et al. 2009), suggests the contribution and close relationships, of two to four diploids to the octoploids (Fig. 2.2). The specific diploid sources of the octoploid genome are still not known but evidence indicates *F. vesca*, *F. mandshurica*, and *F. iinumae* (Senanayake and Bringhurst 1967; Harrison et al. 1997b; Potter et al. 2000; Davis and DiMeglio 2004; Rousseau-Gueutin et al. 2009) as the possible contributors. While some species relationships have been confirmed by crossing studies, others have never been verified. For example, the diploid *F. mandshurica* is assumed to be the ancestor of the tetraploid *F. orientalis* (Staudt 2003). This hypothesis is based on their shared sympodially branching runners, characters absent among species found in the adjacent southwestern China, and their overlapping geographic range in northeastern China (Fig. 2.3). However, phylogenetic analysis (Rousseau-Gueutin et al. 2009; Wambui Njuguna unpublished) does not support this hypothesis.

The diploid *F. nilgerrensis* is speculated to be a diploid ancestor of *F. moupinensis* (Darrow 1966). Interspecific hybridization has resulted in the formation of several species such as *F.* ×*bifera* (*F. vesca* × *F. viridis*) (Staudt et al. 2003), *F. bucharica* (involving diploids, *F. vesca* and *F. viridis*) (Staudt 2006; Rousseau-Gueutin et al. 2009), *F.* ×*ananassa* subsp. *cuneifolia* (*F. virginiana*, *F. chiloensis*) (Staudt 1989), and *F.* ×*bringhurstii* (*F. chiloensis*, *F. vesca*) (Bringhurst and Senanayake 1966).

Limited chloroplast genome variation has created a barrier to phylogenetic resolution of the genus using standard Sanger sequencing (Harrison et al. 1997b; Potter et al. 2000). The low copy nuclear genes, granule-bound starch synthase I (*GBSSI*-2) or *Waxy*, and dehydroascorbate reductase (*DHAR*) were recently used to determine phylogenetic relationships based on sequence comparison in each species (Rousseau-Gueutin et al. 2009). Previously identified relationships such as the basal position of *F. iinumae* in the

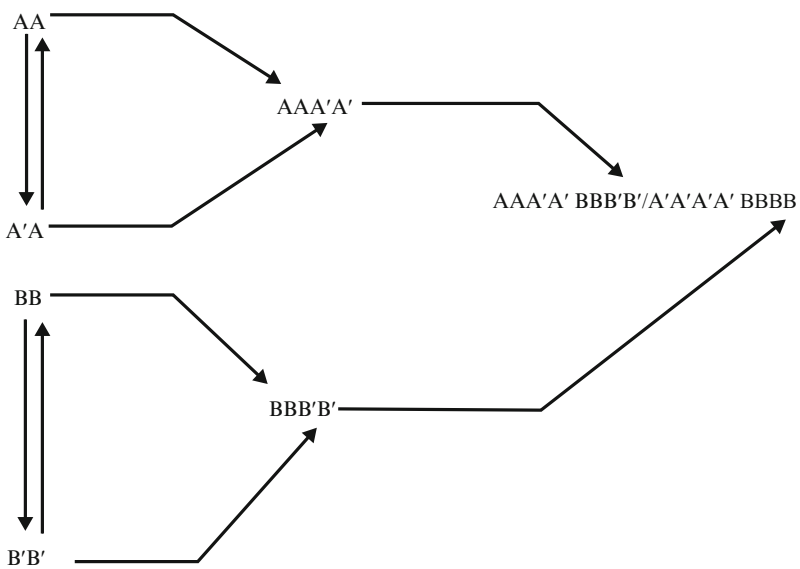

Fig. 2.2 The *Fragaria* octoploid genome model. An illustration of the origin of *Fragaria* octoploid genome modified from Bringhurst (1990) and equivalent to the YYY′Y′ZZZZ/YYYYZZZZ models proposed by Rousseau-Gueutin et al. (2009)

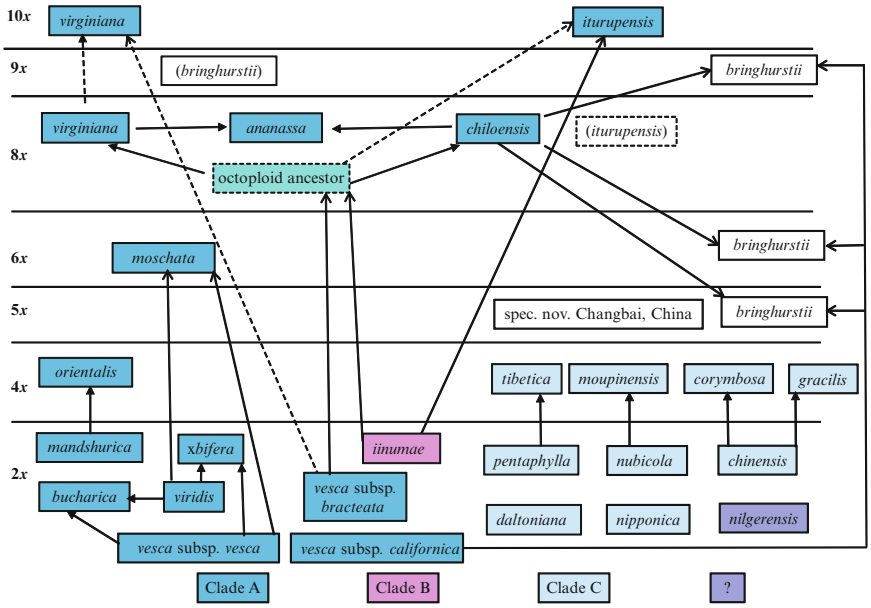

Fig. 2.3 Representation of *Fragaria* species relationships based on nuclear and chloroplast gene sequences and morphological characters (Harrison et al. 1997; Potter et al. 2000; Staudt 2008; Hummer and Hancock 2009; Rousseau-Gueutin et al. 2009). Clades A, B, and C refer to diploid clades determined from nuclear genes *GBSSI*-2 and *DHAR*. They correspond to possible sources of "A" and "B" genomes of the octoploid strawberry. *Dotted lines* indicate hypothetical relationships. *Solid lines* are published relationships

phylogeny and multiple polyploidization events in *Fragaria* (Harrison et al. 1997b; Potter et al. 2000) were confirmed. Analysis of low copy nuclear genes differentiated *Fragaria* diploids into three clades, X (*F. daltoniana*, *F. nilgerrensis*, *F. nipponica*, *F. nubicola*, *F. pentaphylla*), Y (*F. mandshurica*, *F. vesca*, *F. viridis*), and Z (*F. iinumae*) analogous to clades C, A, and B, respectively (Potter et al. 2000), with the octoploid genome originating from clades Y (A) and Z (B) based on the distribution of multiple copies of low copy nuclear genes in the octoploids. The phylogenetic study of Rousseau-Gueutin et al. (2009) is now the most extensive one in *Fragaria* involving a comprehensive species representation and increased phylogenetic resolution. However, there was low resolution of diploid species within clade C supporting recent divergence within the clade and placement of *F. bucharica* low copy genes in different clades (C and A), suggesting hybrid origin of this species or incomplete lineage sorting.

The use of nuclear genes for phylogenetic analysis is complicated by polyploidy and recombination and lineage sorting, making the chloroplast genome an attractive tool for phylogenetic resolution. For the chloroplast genome to be utilized for phylogenetic relationships in *Fragaria*, alternative techniques for finding species-specific identifiers and markers appropriate for phylogenetic resolution need to be explored.

2.3 Conservation Initiatives

In 2008, *Fragaria* genebanks were located in 27 countries and, together with two genebank networks, maintained more than 12,000 strawberry accessions in about 57 locations (Hummer 2008). Roughly half of these accessions represented advanced breeding lines of the cultivated hybrid strawberry. A survey of the private sector indicated that, in addition to the public collections, global private corporations maintained another 12,000 proprietary cultivated hybrids for internal use. Unlike the public collections, however, these private collections were transitory in nature with proprietary genotypes being destroyed after intellectual property rights expire.

Primary collections at national genebanks consisted of living plants, protected in containers greenhouses, or screenhouses or growing in the field. Any plant material grown outdoors cannot be certified as pathogen-

negative. Secondary backup collections were maintained in vitro under refrigerated temperatures. Long-term backup collections of meristems were placed in cryogenic storage at remote locations to provide decades of security. Species diversity was represented by seed lots stored in −18°C or backed-up in cryogenics. Conservation of clonally propagated material, where genotypes were maintained, was more complicated and expensive than storing seeds, where the objective is to preserve genes. The health status of both forms of storage was of primary importance for plant distribution to meet global plant quarantine regulations.

Strawberries are a specialty crop. Limited world resources are available from each government for conservation of cultivated strawberries and their wild relatives. These limited resources constrain the management of strawberry resources in each country (Hummer 2008). Many genebanks are unable to perform pathogen test protocols and maintain pathogen-negative plants that satisfy quarantine requirements. Training on standard protocols for germplasm maintenance is needed for staff of genebanks in developing countries. Coordination of inventory and characterization data between genebanks is also insufficient (Hummer 2009).

In situ preservation of wild strawberries has been limited. The wild species in many regions of the world would be appropriate for such conservation efforts.

2.4 Cytology and Karyotyping

Longly (1926) and Ichijima (1926) performed early cytology of *Fragaria*. They determined that the basic chromosome set was $x = 7$, with four main ploidy levels ranging from diploid to octoploid. Additional decaploid species were since found (Fig. 2.4) (Hummer et al. 2009).

The circumpolarly distributed *Fragaria vesca* was diploid; some Asian species were tetraploid; the European *F. moschata* was the only known hexaploid; and *F. chiloensis* and *F. virginiana* subspecies were octoploid. Subsequent observations of wild and cultivated strawberries confirmed these numbers (Longly 1926; Bringhurst and Senanayake 1966; Nathewet et al. 2007).

Cytologists have also studied *Fragaria* pollen mother cells to examine the phylogenetic relationships between parent and progeny and the genome compositions (Kihara 1930; Scott 1950; Senanayake and Bringhurst 1967: Staudt et al. 2003). Karyotype analyses have been conducted on the wild diploid species, *F. daltoniana*, *F. hayatai* Makino, *F. iinumae*, *F. nipponica*, *F. nubicola*, and *F. vesca*, and octoploid species *F. chiloensis* (Iwastubo and Naruhashi 1989, 1991; Naruhashi et al. 1999; Lim 2000; Nathewet et al. 2009).

Yanagi and his laboratory team have been examining the karyotype analysis in wild strawberries (Nathewet et al. 2009). They examined phylogenetic relationships between species using cluster analysis based on karyotypic similarity. Chromosome morphology in wild diploid strawberries had greater uniformity than that in the tetraploids. Cluster analysis indicated that the diploid and tetraploid species reside in separate clades, with the exception of *F. tibetica*. This tetraploid clustered with the diploid species clade in their analysis.

The hexaploid *F. moschata* clustered with the tetraploid clade. In studies with the octoploids, the size and shape of the Virginian strawberry varied more than that of the beach strawberry. Each of these octoploid species was separated into distinct clades. The Asian *F. iturupensis* grouped with the Virgianian strawberry clade. It is also similar in morphology to *F. virginiana* subsp. *glauca*.

2.5 Classical and Molecular Genetic Studies

Many strawberry cultivars have been grown around the world and new varieties appear at frequent intervals (Nielsen and Lovell 2000). The continued introduction of strawberry cultivars to the market increases the need for reliable methods of identification and genetic diversity assessment (Degani et al. 2001). In addition, verification of strawberry cultivars is essential for growers and plant breeders to protect breeders' rights (Garcia et al. 2002).

Verification is especially important in a clonally propagated crop like strawberry where one original plant of an economically important cultivar can be easily used to produce a large number of plants (Gambardella et al. 2001). Strawberry cultivars have been identified using morphological traits (Nielsen and Lovell 2000) and molecular markers (Levi et al. 1994; Congiu et al. 2000; Degani et al. 2001; Garcia et al. 2002; Shimomura and Hirashima 2006; Govan et al. 2008; Brunnings et al. 2010). Molecular marker techniques for analysis of strawberries include isozymes

Fig. 2.4 Chromosome separation at metaphase in a *Fragaria iturupensis* Staudt root tip cell (Hummer et al. 2009); *bar* represents 5 μm

and hybridization-based and PCR-based DNA markers and complement the use of morphological markers in germplasm characterization.

2.5.1 Morphological Identification of Strawberries

Morphological characterization in strawberry involves recording variation in habit, leaf, flower, and fruit traits (Dale 1996). Morphological characters traditionally identified crop species and varieties (Nielsen and Lovell 2000) and have been used in Argentina to certify cultivar identity in strawberry (Garcia et al. 2002). In the United States and Europe, morphological markers are used in addition to isozyme markers in plant patent descriptions (Nielsen and Lovell 2000).

Morphological characters vary with age, time of year, production enhancement regimes, and cultivation methods (Degani et al. 2001). These characters are subjective and can vary between reports and environments (Bringhurst et al. 1981). In an identification study of strawberry cultivars from Argentina, morphological

characters were insufficient to distinguish between three genotypes of "Pajaro" that were found to be polymorphic using molecular markers (Garcia et al. 2002). A set of morphological characters to uniquely identify strawberry cultivars (Nielsen and Lovell 2000) includes leaf morphology, leaf length and breadth, leaf base shape, teeth base shape, petal spacing, petal length and base, calyx:corolla (length ratio), fruit size, fruit length and breadth, fruit shape, band without achenes, insertion of achenes, insertion of calyx, and calyx size. In most cultivar identification cases, especially those dealing with infringement of breeders' rights, only the fruit, and not the whole plant, is available.

In a study by Kunihisa et al. (2003), strawberry imports to Japan were suspected to be mixed with Japanese varieties not licensed for production in other countries. Only the fruit was available for identity verification. Fruit processing and canning industry sales depend on marketing released varieties. Morphological markers are the traditional technique for distinguishing cultivars; however, they can sometimes result in ambiguity for identification (Chavarriaga-Aguirre et al. 1999; Dangl et al. 2001; Abu-Assar et al. 2005). This suggests the need for additional forms of identification. DNA extraction kits suitable for processed fruit are now available (for example Genetic ID, Inc. Fairfield, IA), which allow identification of cultivars using molecular markers.

Despite disadvantages associated with morphological character traits, they have proved useful in breeding programs and germplasm repositories. Morphological traits help to group plants with similar qualitative and quantitative traits (Brown and Schoen 1994). However, lack of discrimination between individuals is explained by the plasticity of morphological markers (Degani et al. 2001).

2.5.2 Isozymes

Isozymes are enzymes with different amino acid sequence that catalyze the same reaction. Isozymes exhibit different electrophoretic mobility, and different forms are easily distinguished. Isozyme markers were the first molecular markers to be developed. Their use in strawberry dates to the late 1970s (Hancock and Bringhurst 1979). Isozymes were used to determine adaptive strategies of 13 *F. vesca* (diploid) and 19 octoploid *Fragaria* populations from California using two enzyme systems, phosphoglucoisomerase (PGI) and peroxidase (PX). In both the diploid and octoploid species, a high genetic differentiation was observed that depended on the site of collection. The association was attributed to variations in catalytic properties of the isozymes expressed under different environmental conditions. This illustrates the sensitivity of isozymes to the environment, even within the same species. Nevertheless, isozymes were used in strawberry for cultivar identification (Nehra et al. 1991) and in linkage analysis (Williamson et al. 1995).

Like morphological markers, isozyme variation can depend on the environment or age of the plant (Hancock and Bringhurst 1979). Isozymes also exhibit low polymorphism due to the limited number of detected alleles (Khanizadeh and Bélanger 1997; Nehra et al. 1991). In a study using three enzyme assays, PGI, leucine aminopeptidase (LAP), and phosphoglucomutase (PGM), Gálvez et al. (2002) characterized 24 strawberry cultivars.

Thongthieng and Smitamana (2003) used four enzyme systems (malate dehydrogenase, malic enzyme, leucine amino peptidase, and diaphorase) to analyze strawberry progeny from alternate crosses of four parental lines. They could not identify hybrid lines at either 90 or 95% similarity levels. They recommended using a larger number or another set of enzyme systems for fingerprinting strawberry cultivars. Gálvez et al. (2002) and Gambardella et al. (2001) suggested that isozymes could be more effectively applied for verification of cultivars and inferring relationships between groups of cultivars as opposed to fingerprinting.

2.5.3 DNA-Based PCR Markers

2.5.3.1 Random Amplified Polymorphic DNA

Random amplified polymorphic DNA (RAPD) markers were the first PCR-based method used for cultivar identification (Williams et al. 1990). These markers are well-distributed throughout the genome, have a rapid non-radioactive detection procedure (Gidoni et al. 1994), and do not require DNA sequence information prior to primer synthesis (Williams et al. 1990; Congiu et al. 2000). RAPD markers are expressed as dominant traits; the amplification with random

markers proceeds only in the presence of a pair of sequences homologous to that of the primer (~10 bp long) on either one or both homologous chromosomes (Zhang et al. 2003). This molecular marker was adopted as a tool that overcame limitations observed with isozymes such as sensitivity to the environment and the low number of detected alleles (Arulsekar et al. 1981; Hancock et al. 1994; Levi et al. 1994).

Identification of closely related strawberry varieties is important in the protection of breeders' rights. A perfect example of the protection of breeders' rights using molecular markers was in the settling of a lawsuit where unambiguous identification of a cultivar, "Onebor" (MarmoladaTM), was required by court decree (Congiu et al. 2000). RAPDs were able to distinguish 13 clones of the cultivar "Onebor" (MarmoladaTM) from a group of 31 plants.

The use of RAPDs was extended to distinguishing wild species populations in North and South America. These molecular markers partitioned most of the variation among plants within *F. virginiana* and *F. chiloensis* populations from North America using analysis of molecular variance (AMOVA) (Harrison et al. 2000) but were unable to discriminate among the four subspecies of *F. virginiana* (Harrison et al. 1997a). Morphological markers, however, distinguished among the four subspecies of *F. virginiana* and grouped them into different provenances. Even though RAPD markers could not distinguish between *F. virginiana* subsp. *virginiana* and subsp. *glauca*, they indicated a high within-population variation.

In another study, RAPD-based cluster analysis separated the North American (*F. chiloensis* subsp. *lucida* and subsp. *pacifica*) from the South American plants (*F. chiloensis* subsp. *chiloensis*) but did not separate the two North American subspecies (Porebski and Catling 1998). These studies suggest that in strawberries, random molecular markers were better suited for discriminating between genotypes (individuals) rather than for revealing relationships among wild populations (Harrison et al. 1997a, 2000).

Low levels of reproducibility within and between laboratories, a low level of polymorphism, as well as the inability to detect allelism reduces the usefulness of RAPDs for plant fingerprinting and identification. Low reproducibility results from amplification of DNA using short random primers that do not specifically bind the template (Garcia et al. 2002). Irreproducibility can also result from selecting a subset of the bands on agarose gels, usually the more intense ones (Gidoni et al. 1994; Hancock et al. 1994), resulting in variable scores of the same cultivars from different laboratories. Gidoni et al. (1994) observed consistent and significantly lower amplification with two primer–individual combinations that they attributed to mismatches in primer binding or presence of secondary structures in the DNA hindering PCR. Detection of polymorphism and reproducibility using RAPDs can be increased by screening a large set of random primer pairs, carrying out reactions in replicate and maintaining stringent conditions (Gidoni et al. 1994; Hancock et al. 1994; Jones et al. 1997). For example, Porebski and Catling (1998) selected 12 of 100 RAPD primers that were 100% reproducible in replicates of the 35 samples used in the genetic diversity study of North and South American *F. chiloensis* subspecies. Garcia et al. (2002) repeated amplifications four times with a set of 13 RAPD primers to discriminate among eight accessions to ensure reproducibility and avoid artifacts. They also used polyacrylamide gels to increase the resolution of amplified fragments, which resulted in 37 cultivar-specific bands in only three of those 13 primers. Landry et al. (1997) verified amplification profiles and polymorphism in 75 strawberry cultivars and lines using DNA from two independent microextractions, while Levi et al. (1994) ensured reproducibility by repeating reactions two or three times with eight RAPD primers to check the genetic relatedness among nine strawberry clones.

Modifications of the RAPD technique in an effort to minimize disadvantages of using short random primers led to the development of two molecular markers, namely cleaved amplified polymorphic sequences (CAPS) and sequence characterized amplified regions (SCARs).

CAPS markers are developed after PCR to reveal variation among individuals of interest. Following PCR amplification of a locus, restriction enzymes are used to cleave the amplified product and reveal polymorphisms resulting from mutations in restriction sites in the different individuals. In strawberry, CAPS markers were developed by Kunihisa et al. (2003) for verification of the identity of strawberry cultivars imported into Japan. Polymorphism detected was reproducible irrespective of DNA extraction method, DNA source tissue (leaves, sepals, or fruit), or laboratories (four different researchers). Six CAPS markers were developed in the study and five

of these were sufficient to distinguish 14 cultivars from Japan.

The development of CAPS markers can be expensive because it involves extensive sequencing (if sequence information is unavailable) and screening for restriction enzyme-genomic locus combinations that yield polymorphic products. In the study by Kunihisa et al. (2003), out of 156 restriction enzyme-genomic locus combinations only nine were polymorphic, a discrepancy explained by the insufficient DNA sequence information.

SCARs result from cloning and sequencing a RAPD PCR product, designing longer primers (~20 bp in length) from the ends of the sequenced amplified product, and using these primers for PCR (Paran and Michelmore 1993). The SCAR primers are longer than RAPD primers and subsequently amplify a specific DNA fragment under highly stringent annealing temperatures. A high reproducibility of SCARs results from lack of mismatching in the priming site during amplification experienced when using RAPD primers (Garcia et al. 2002). One of seven RAPD markers developed by Haymes et al. (1997) linked to a red stele resistance gene, *Rpf1*, in strawberries was converted to a SCAR marker (Haymes et al. 2000) to increase the reproducibility of screening for resistant strawberry cultivars. Two SCAR markers were also developed that are linked to the *Rca*2 gene-encoding resistance to anthracnose (*Colletotrichum acutatum*) pathogenicity group 2 (Lerceteau-Köhler et al. 2005). The drawback associated with the two modifications, CAPS and SCARs, is the need for the laborious cloning and DNA sequencing for their development.

2.5.3.2 Amplified Fragment Length Polymorphism

The amplified fragment length polymorphism (AFLP) technique first described by Vos et al. (1995) involves (1) restriction of DNA template (2) ligation of oligonucleotide adapters (3) pre-amplification, which involves amplification of the DNA with primers that have only one selective nucleotide thus reducing the number of DNA fragments generated and (4) selective amplification of sets of restriction fragments, which are then visualized on sequencing gels or by capillary electrophoresis. AFLP reveals polymorphisms as the presence or absence of a restriction fragment rather than length differences and is consequently scored as a dominant marker (Vos et al. 1995).

Due to the dependence on restriction and ligation, AFLP requires a high level of DNA purity (Arnau et al. 2001), and degraded or contaminated DNA may result in incomplete restriction digestion (Perry et al. 1998) that does not reflect the true polymorphism present (Vos et al. 1995). High reproducibility and a large number of polymorphic products are the two main advantages of AFLP markers over RAPDs (Schwarz et al. 2000). The first report of the use of AFLP in strawberry was by Degani et al. (2001) who compared the genetic relationships based on pedigree, RAPD (Degani et al. 1998), and AFLP data in 19 strawberry cultivars. Nine cultivar-specific AFLP bands were identified from a total of 228 bands while 35 (15.4%) were polymorphic. These 35 polymorphic markers distinguished the 19 strawberry cultivars. A surprising result was the higher correlation of pedigree data coefficients with RAPD rather than with AFLP similarity coefficients. This result was explained by the possible even distribution of the RAPD markers used across the strawberry genome (Degani et al. 2001). The AFLP technique was also used to identify 19 strawberry genotypes from Poland (Tyrka et al. 2002). Using one restriction enzyme, *Pst*I, they obtained a total of 129 bands of which 22 (17%) were polymorphic.

As with RAPDs, AFLPs were converted to SCAR markers that were useful in strawberry breeding. By screening 179 strawberry individuals from a cross of the resistant "Capitola" and susceptible "Pajaro" with 110 *Eco*RI/*Mse*I AFLP combinations, four AFLP markers were found to be linked in coupling phase to the *Rca*2 gene responsible for resistance to anthracnose (Lerceteau-Köhler et al. 2005). Two of these markers were converted into SCARs. There was a high (81.4%) level of accuracy in the detection of resistant/susceptible genotypes from a group of 43 cultivars. These developed SCAR markers are useful in the detection of resistance in a marker-assisted selection (MAS) program since they are easier to detect as opposed to the large number of amplified products with the AFLP technique.

2.5.3.3 Microsatellites or Simple Sequence Repeats

Microsatellites, also known as simple sequence repeats (SSRs), are stretches of tandemly repeated di-, tri-, or tetra-nucleotide DNA motifs that are abundantly dispersed throughout most eukaryotic genomes

(Powell et al. 1996; Zhu et al. 2000). These short tandem repeats are found in non-coding and genic regions of the genome (Varshney et al. 2005).

In strawberry, ten SSRs were first developed from genomic sequences of *F. vesca* "Ruegen" (James et al. 2003). Owing to the advantages associated with SSRs, including codominance, multiallelism, and high rates of polymorphism and reproducibility, the number of published *Fragaria*-derived SSRs has continued to increase. Over 900 *Fragaria*-derived SSR primer pairs are currently available for molecular studies. These SSRs were developed from genomic libraries (Ashley et al. 2003; James et al. 2003; Sargent et al. 2003; Cipriani and Testolin 2004; Hadonou et al. 2004; Lewers et al. 2005; Monfort et al. 2006; Spigler et al. 2008, 2010), GenBank sequences (Lewers et al. 2005), or EMBL sequences and expressed sequence tags (EST) (Folta et al. 2005; Bassil et al. 2006a, b; Keniry et al. 2006; Spigler et al. 2008, 2010; Zorrilla-Fontanesi et al. 2010). These published SSRs were developed from the diploid *F. vesca* (James et al. 2003; Cipriani and Testolin 2004; Hadonou et al. 2004; Monfort et al. 2006; Bassil et al. 2006b; Zorrilla-Fontanesi et al. 2010), diploid *F. viridis* (Sargent et al. 2003), octoploid *F. virginiana* (Ashley et al. 2003; Spigler et al. 2010), and the domestic strawberry *F.* ×*ananassa* (Bassil et al. 2006a; Gil-Ariza et al. 2006; Zorrilla-Fontanesi et al. 2010).

Most SSR primer pairs were developed from the cultivated strawberry, *F.* ×*ananassa*, followed by *F. vesca*, *F. viriginiana*, and *F. viridis*. Each of the studies, except for two (James et al. 2003; Keniry et al. 2006), tested for cross transferability of developed SSRs to species other than the focal species. From one to 15 *Fragaria* species (excluding the focal species) were used to check cross species SSR transferability in the remaining publications. These studies have reported high levels of cross-species transferability within *Fragaria*. The highest levels of amplification were observed in the cultivated species, *F.* ×*ananassa*, in studies where it was the focal (Cipriani and Testolin 2004; Hadonou et al. 2004; Bassil et al. 2006a) and the non-focal (Lewers et al. 2005; Bassil et al. 2006b) species. Amplification products were observed in *F.* ×*ananassa* and *F. chiloensis* from microsatellites developed for *F. virginiana* (Ashley et al. 2003). Thirty-seven primer pairs developed from *F.* ×*ananassa* "Strawberry Festival" revealed between 89% amplification in *F. vesca* to 100% amplification in *F. chiloensis* and *F. virginiana* (Bassil et al. 2006a). Hadonou et al. (2004) reported 77–100% transferability of 31 SSRs from *F. vesca* to other diploids and to the *Fragaria* octoploids, respectively. With 20 microsatellite primer pairs developed from *F. vesca*, 95% transferability was observed to *F.* ×*ananassa* (Cipriani and Testolin 2004). This transferability of SSRs between the octoploids and the diploids presents an advantage in comparative mapping and synteny studies in *Fragaria* (Rousseau-Gueutin et al. 2008).

To date, microsatellite markers in *Fragaria* have been used for cultivar identification (Shimomura and Hirashima 2006), fingerprinting (Govan et al. 2008; Brunnings et al. 2010; Njuguna 2010a; Njuguna and Bassil 2008), genetic diversity analysis (Njuguna et al. 2009b, 2010), and linkage mapping (Sargent et al. 2004, 2006, 2009; Nier et al. 2006; Spigler et al. 2008, 2010). Shimomura and Hirashima (2006) were able to distinguish ten popular Japanese strawberry cultivars using two SSRs developed from "Toyonoka". The development of SSRs to distinguish these Japanese strawberries was triggered by the infringement of Japanese strawberry breeders' rights. The first microsatellite fingerprinting set for cultivated strawberry was developed by Govan et al. (2008) at East Malling Research (EMR) in the UK. A set of ten SSR primer pairs, flanking dinucleotide repeats, was selected from 104 that were tested. This set can be multiplexed, reducing cost and time for conducting experiments, and was evaluated in 60 octoploid accessions. The accessions included 56 *F.* ×*ananassa* cultivars and four wild octoploid *Fragaria* species representatives. The multiplex set was able to discriminate among the genotypes tested and a standard cultivar set was identified that will facilitate the harmonization of allele calling among laboratories. Nine of the ten SSRs in this fingerprinting set was used to fingerprint 26 cultivars and advanced selections from the University of Florida strawberry breeding program (Brunnings et al. 2010). More recently, a reduced fingerprinting set of four SSRs was selected from 91 primer pairs based on multiplexing ability, reproducibility in different labs, ease of scoring, high polymorphism in the domestic strawberry and its immediate octoploid progenitors, and ability to identify each accession from 22 species maintained at the Corvallis National Clonal Germplasm Repository (Njuguna and Bassil 2008; Njuguna 2010a). This reduced set consisted of three of the ten SSRs recommended by Govan et al. (2008) and an additional trinucleotide repeat-containing SSR. Public

molecular databases of genotypic data generated with universal SSRs and using reference genotypes to harmonize genotype calling will provide a valuable resource for cultivar identification and quick detection of misidentified accessions to the strawberry breeder, grower, nursery, and research communities.

The availability of SSR markers that were identified to amplify in *Fragaria* species of interest through cross-transference studies allows their use in population and diversity analysis. We identified 20 such primer pairs out of 91 SSRs in *F. iinumae* and *F. nipponica* and used them to evaluate genetic diversity and population structure of wild Asian diploid species collected from Hokkaido, Japan (Njuguna et al. 2009b, 2010). A model-based Bayesian clustering among accessions representing the two species groups in the program STRUCTURAMA™ (Huelsenbeck and Andolfatto 2007) identified ten groups, seven of *F. iinumae* and three in *F. nipponica*, which represent the diversity of these species collected from 22 geographical locations in their native habitat. These representative groups of diploids also reflect the population structure: high population structure of the self-compatible *F. iinumae* is represented by seven groups while low population structure of the self-incompatible *F. nipponica* is captured by three groups. Preservation of this wild germplasm based on diversity (ten groups) as opposed to the traditional method according to geographical location (22 localities) is more accurate and efficient and will allow the capture and use of the available diversity in this Hokkaido collection.

2.5.4 Linkage Mapping in Strawberry

A strawberry linkage map was first constructed from a *F. vesca* F_2 population obtained from a cross between "Baron Solemacher" (BS), a highly homozygous inbred line, and WC6, a wild accession (Davis and Yu 1997). The resulting map was 445 cM in length and contained a total of 79 markers including 75 RAPD markers, an alcohol dehydrogenase locus (*Adh*), phosphoglucose mutase (*Pgi*-2) isozyme locus, shikimate dehydrogenase (*Sdh*) isozyme locus, and the runnering locus. An additional locus, *F. vesca* fruit color locus (c) that did not segregate in the F_2 population in the studies of Davis and Yu (1997) was mapped based on its previously established linkage to the *Sdh* locus (Williamson et al. 1995). Among the 75 RAPD markers mapped to the *F. vesca* map, 11 were identified as codominant. Codominant RAPD markers were identified after detection of heteroduplex bands following PCR with mixed templates (mixed parent DNA and/or parent DNA mixed with F_2 progeny DNA), a method described by Davis et al. (1995).

The first genetic linkage map for the octoploid strawberry, *F.* ×*ananassa*, was constructed using AFLP markers (Lerceteau- Köhler et al. 2003). Two putative genes, alcohol transferase (AAT) and the dihydroflavonol reductase (DHFR), were also mapped onto the octoploid map. A full-sib progeny consisting of 113 individuals obtained from a cross of "Capitola" and CF1116 (a reference from the Research and Interregional Experimentation Centre of Strawberry, Ciref, France), was used as the mapping population. Single dose restriction fragments (SDRFs) (a fragment found in only one of the parents) were used to study repulsion phase linked markers, while a pseudo-testcross configuration was used to develop two linkage maps (a female and a male linkage map). A total of 235 and 280 SDRFs were mapped on the female (1,604 cM) and male (1,496 cM) maps, respectively, covering 43 cosegregating groups in each of the maps. AFLP markers were also used to build a genetic map and identify quantitative trait loci (QTL) for day-neutrality in a population of 127 progeny of the day-neutral (DN) "Tribute" and the short-day (SD) "Honeoye" (Weebadde et al. 2008). The map was 1,541 cM in length with 43 linkage groups. Out of the eight QTLs found that were either location-specific or shared among locations, none explained >36% of the phenotypic variation, indicating that the inheritance of day-neutrality is likely a polygenic trait in strawberry.

Dominant markers such as RAPDs and AFLPs are not locus-specific and are therefore not easily transferable to other related genomes of similar species or populations (Sargent et al. 2004). The low transportability of dominant markers influenced the use of transferable locus specific markers to create a linkage map to be used as a framework for future mapping studies in *Fragaria*. Sargent et al. (2004) mapped 68 SSRs, six gene-specific markers and one SCAR marker in an F_2 population of 94 seedlings obtained from an interspecific cross of diploid *F. vesca* × *F. bucharica* L (FV × FB). Seventeen of the markers were scored as dominant markers (presence/absence) because they occurred in

only one of the parents while 58 were codominant. Mapping of SSRs and gene-specific markers creates a good framework for future mapping studies, which include marker-assisted breeding and selection in the cultivated strawberry, positional cloning, and synteny studies that can transfer marker information from the diploid to octoploid relatives within a genus (Davis and Yu 1997; Sargent et al. 2004). SSRs were added to the BS × WC6 (Davis et al. 2006) and to the reference map of Sargent et al. (2004), increasing its marker density by 149% (Sargent et al. 2006). To confirm the utility of the reference map as a standard in mapping studies, Nier et al. (2006) developed a reduced linkage map using SSR and gene-specific markers constructed from a wide interspecific backcross between two *Fragaria* species, *F. vesca* × [*F. vesca* × *F. viridis*]. In this comparative study, marker order was conserved between both maps on three of the seven linkage groups; genetic distances were similar to those on the reference map. Differences in marker order were attributed to the distant relationship of *F. viridis* to the diploid species *F. bucharica* and *F. vesca* as well as to the octoploid *F.* ×*ananassa* (Potter et al. 2000). A significant reduction in recombination frequencies between markers (and therefore mapping distances) was observed when compared to the reference map. This difference was attributed to a decrease in the frequency of chiasmata formation due to reduced homology between the homeologous chromosomes of the parental species used (Chetelat et al. 2000). Nier et al. (2006) concluded that the reference map generated by Sargent et al. (2004) was useful in generating transferable maps within the *Fragaria* genus.

The diploid reference map (FV × FB) of Sargent et al. (2004) was used to select markers for mapping in an F_1 population from a cross of *F.* × *ananassa* cultivars, Red Gauntlet and Hapil (RH × H) (Sargent et al. 2009). The use of transferable SSR markers facilitated comparison of the two maps derived from FV × FB and RH × H crosses, which revealed complete synteny apart from a possible duplicated region observed in the octoploid map. The observed synteny will be useful for future comparative mapping studies. The authors attributed the possible duplicated markers in the octoploid genome to either a consequence of ancient polyploidization event or duplication in one of the diploid progenitors of the polyploids.

An SSR-based linkage map was recently constructed in *F. virginiana* where also sex determination was mapped as two qualitative traits, male and female function (Spigler et al. 2008, 2010). The resultant maternal and paternal maps comprised 33 and 32 linkage groups, 319 and 331 markers, respectively. Twenty-eight chromosomes of *F. virginiana* were assembled into seven groups of four homeologous chromosomes by SSR commonality and comparison to the diploid *Fragaria* map (Sargent et al. 2006, 2008). Both sex expression traits mapped to the same linkage group (LG6C-m, p), which shared nine SSRs with the diploid LG 6, indicating autosomal origin of this "proto-sex" chromosome. Limited recombination occurs between two linked loci carrying the male and female sterility mutations that control sex determination in *F. virginiana*. Evidence of recombination between these two loci, an important hallmark of incipient sex chromosomes, suggests that *F. virginiana* might contain the youngest sex chromosome in plants and provide a novel model system for the study of sex chromosome evolution. Comparison of this map to previously published diploid strawberry maps (Davis et al. 2006; Sargent et al. 2006, 2008) that contained SSR markers in common indicated some conservation of linkages, some rearrangements in the octoploid genome between the diploid LG 1 and LG 6 to create the linkage groups present in the octoploid. Fine mapping and additional comparative analysis will allow better understanding of the evolution of octoploidy and sex determination in strawberry.

2.5.5 DNA Barcoding

DNA barcoding, often referred to as barcoding, was proposed as a practical method to identify species by variation in short orthologous DNA sequences from one or a small number of universal genomic regions. In animals, a 600 bp sequence at the 5′ end of the mitochondrial gene, cytochrome c oxidase 1 (*COX*1), was used successfully due to its rapid mutation rate in birds (Hebert et al. 2004a), fish species (Ward et al. 2005), and skipper butterflies (Hebert et al. 2004b). Limited variation in sequence and rapid change of structure in the mitochondrial genome of plants (Chase et al. 2005; Rubinoff et al. 2006) led to the exploration of other genomes for an alternative DNA barcode region. A two DNA barcode system for plants involving the nuclear internal transcribed spacer (nr*ITS*) and the chloroplast *psb*A–*trn*H intergenic

spacer was proposed (Kress et al. 2005). The DNA barcoding technique is simple and can be utilized for routine initial screening of species collections in genebanks. If successful, DNA barcoding could enhance the efficiency of germplasm management by providing a quick method of identification and classification of species. These two proposed DNA barcoding regions, *psb*A–*trn*H and nr*ITS*, were tested in *Fragaria* species preserved at the USDA-ARS repository in Corvallis, Oregon (Njuguna et al. 2009a). The "barcoding gap", between within species and between species variation, required for discriminating between species was absent, preventing identification of *Fragaria* species. DNA barcoding did not work for identifying *Fragaria* species and we believe that it will not identify taxa with little genetic variation.

2.5.6 Chloroplast Genome Markers

The size of the chloroplast genome, its non-recombinant nature, and high sequence conservation reduces the complexity of analysis and interpretation of results. This maternally inherited genome in *Fragaria* was exploited for phylogenetic relationships and to resolve unanswered evolutionary questions. Chloroplast molecular markers included restriction fragment length polymorphisms (cpRFLP) (Harrison et al. 1997a, b), nucleotide sequences (Lin and Davis 2000; Lundberg et al. 2009; Mahoney et al. 2010; Njuguna et al. 2009a), simple sequence repeats (cpSSRs) (Njuguna 2010b), as well as almost complete genome and are listed in Table 2.2.

Phylogenetic analysis using chloroplast RFLPs (Harrison et al. 1997a, b) and chloroplast nucleotide sequences (Potter et al. 2000; Njuguna et al. 2009a) have resulted in unclear relationships due to the limited variation in this genome. Harrison et al. (1997a, b) used chloroplast DNA RFLP from nine species, and Potter et al. (2000) used the nuclear internal transcribed spacer (nr*ITS*) region and the chloroplast regions, *trn*L intron and the *trn*L–*trn*F spacer in 14 species. These two studies resulted in low resolution of strawberry species relationships that was speculated to result from small taxon sampling and low amount of sequence variation in the genome test regions (Rousseau-Gueutin et al. 2009). Compared to chloroplast sequences of other Rosaceae members, *Fragaria* seems to have limited variation.

Microsatellites in the chloroplast genome (cpSSRs) mostly "A" or "T" mononucleotide repeats, though less variable than nuclear SSRs, have been used in numerous plant genetic studies. The non-recombining nature has been exploited for the design of universal primer pairs flanking chloroplast SSRs distributed across the chloroplast genome. Four universal cpSSRs, ccmp2, ccmp5, ccmp6, and ccmp7 developed in *Nicotiana tabacum* by Weising and Gardner (1999) were tested in 96 accessions representing 22 *Fragaria* species. Exploitation of these highly variable regions revealed moderate genetic diversity of these markers in strawberry (mean 0.54) (Njuguna 2010b). Sequencing of cpSSR alleles revealed lack of conservation and even loss (in ccmp6) of the microsatellite repeat in addition to size homoplasy, thus making use of size variation in determining haplotype identity of these universal markers incorrect for inferring phylogenetic inference in *Fragaria*.

For efficient use of limited chloroplast sequence divergence, a large scale sequencing study would be required, now possible with high-throughput sequencing platforms such as Illumina 1G/Solexa (Illumina

Table 2.2 List of *Fragaria* chloroplast genome sequences used, the number of species from which the sequences were obtained, and the reference

Chloroplast sequence	# *Fragaria* species	Reference
*trn*L intron and *trn*L–*trn*F	14	Potter et al. (2000)
*rps*18–*rpl*20 and *psb*J–*psb*F	4	Lin and Davis (2000)
*trn*L–*trn*F and *trn*S–*trn*G	3	Lundberg et al. (2009)
*psb*A–*trn*H	21	Potter et al. (2000) and Njuguna et al. (2009a)
YCF2/ORF2280 3′-ORF79 and *ndh*B 5′ exon to *rps*7 5′ end	18	Njuguna et al. (2009a)
Chloroplast SSR sequences		
5′ to *trn*S, 3′ to *rps*2, ORF77-ORF82, *atp*B–*rbc*L	18	Njuguna (2010b)

Inc., San Diego, CA), 454 Life Sciences GS 20 (454 Life Sciences, Branford, CT) and/or SOLiD (Applied Biosystems, Foster City, CA). Sequencing multiple small genomes by taking advantage of the high sequencing depth of high-throughput sequencing platforms was recently tested (Cronn et al. 2008).

Sequencing of complete plastome sequences using Illumina technology was demonstrated in pines (Cronn et al. 2008). A range of 88–94% coverage of the chloroplast genome was obtained from 36 bp single read sequencing in one lane of the Solexa flow cell of four different barcoded PCR products of pine species. Multiplexing of small organellar genomes in single lanes utilizes the sequencing depth, of up to 40 million clusters per flowcell (Morozova and Marra 2008). We used three different approaches for sequencing *Fragaria* chloroplast genomes with the Illumina Genome Analyzer: PCR amplification, physical chloroplast isolation, and plastome assembly from low coverage genomic sequencing (Njuguna 2010a, b). Low coverage genomic sequencing was identified as the most efficient approach for obtaining complete chloroplast genome sequences. Preliminary analysis of the sequencing data confirmed maternal inheritance of the chloroplast in *Fragaria* and identified *F. vesca* subsp. *bracteata* as the maternal donor to the octoploids (*F. chiloensis*, *F. virginiana* and *F.* ×*ananassa* subsp. *cuneifolia*) and the decaploid, *F. iturupensis*. Complete chloroplast genome sequences will be useful in revealing polymorphisms in plant species groups that have little or no detected variation such as *Fragaria* (Harrison et al. 1997a, b; Potter et al. 2000) facilitating species relationship resolution.

2.5.7 Mitochondrial DNA Markers

Mitochondrial DNA (mtDNA) has received the least attention compared to nuclear and plastid genomes. Mahoney and Davis (2010) described the first mtDNA markers in the *matR* gene region in *Fragaria*. This marker provided evidence that mtDNA was transmitted maternally in two interspecific crosses, and that diploid *F. iinumae* is the likely mtDNA donor to the octoploid species *F. chiloensis* and *F. virginiana* but not to decaploid *F. iturupensis*. Additional *Fragaria* mtDNA sequences were obtained by assembling a 67 kb mtDNA contig from Illumina 36 bp paired-end reads of "Pawtuckaway", providing a basis for the development of additional mtDNA markers.

2.6 Genomics Resources Developed

The diploid *F. vesca* was adopted as a model perennial representative of the Rosaceae family. Resources were developed for two other model species for the Rosaceae, apple (*Malus* ×*domestica*) and peach (*Prunus persica*) (Shulaev et al. 2008). Advantages of *F. vesca* include its small genome size (~200 Mbp/1C), short generation time, transformation efficiency, self-compatibility, and abundant seed production (Shulaev et al. 2008). The following gives a brief overview of the genomic resources now available for strawberry and is not meant to be exhaustive.

Since July 2005, when approximately 7,000 genomic and cDNA sequences were listed in GenBank (Davis et al. 2007), the number of *Fragaria* entries has increased to 60,429 nucleotide sequences in April, 2010. The majority of these sequences are ESTs, which account for 58,573, mostly from *F. vesca* (47,743), followed by *F.* ×*ananassa* (10,830).

The Genome Database for Rosaceae (GDR) is an important resource for the Rosaceae research community that was initiated in response to the growing availability of genomic data for peach and has benefited the strawberry community. GDR is a curated and integrated web-based relational database that provides centralized access to Rosaceae genomics and genetics data and analysis tools to facilitate cross-species comparison and use of this data (Jung et al. 2004, 2008). Current strawberry resources enabled by initial funding by the NSF Plant Genome Program in 2003 are available at *http://www.rosaceae.org/node/31* and include: Two diploid linkage maps [FV × FN diploid reference map (Sargent et al. 2006) and 815 × 903 BC map (Nier et al. 2006)] viewed and compared through the comparative map viewer CMap; A fourth assembly of unigenes from publicly available ESTs of diploid and polyploid strawberry that contains a total of 13,896 putative unigenes; and lists and links to currently funded strawberry projects and to other public strawberry databases. One *F. vesca* and another *F.* ×*ananassa* assembly from the National Center for Biotechnology Information (NCBI) nucleotide and EST sequences are available at The Institute for Genomic Research (TIGR) Plant Transcript

Assembly website (Childs et al. 2007) and can be downloaded at *http://plantta.jcvi.org/cgi-bin/plantta_release.pl*. Sequences from *F. vesca* were assembled into 4,825 contigs and 8,624 singlets while those from *F.* ×*ananassa* include 358 contigs and 4,778 singlets.

Large insert bacterial artificial chromosome (BAC) libraries of strawberry have been reported as constructed or under construction (Davis et al. 2007; Shulaev et al. 2008). However, a fosmid library has been constructed from *F. vesca* subsp. *americana* "Pawtuckaway" (Davis et al. 2007) and used for GeneTrek analysis (Pontaroli et al. 2009). Assembly of the resulting ~1 Mb of the nuclear genomic DNA identified 158 genes arranged in gene-rich regions and intermixed with transposable elements (TEs). Of over 30 classified repeat families, long terminal repeat (LTR) retrotransposons were the most abundant in *F. vesca* and comprised ~13% of the genome sequence analyzed. This study predicted the *F. vesca* genome to contain at least 16% of its content in TEs, about 30,500 protein-encoding genes, and over 4,700 truncated gene fragments (Pontaroli et al. 2009).

In addition to nuclear isozyme and PCR-based RAPD, SCAR, CAPS, AFLP, and SSR markers described in the previous section, a limited number of gene-specific markers exist in strawberry (Davis and Yu 1997; Deng and Davis 2001; Sargent et al. 2007). Sequence tag sites (STS) were developed for the alcohol dehydrogenase *ADH* gene (Davis and Yu 1997), five genes in the anthocyanin biosynthesis pathway and one associated transcription factor (Deng and Davis 2001) and 24 genes of known function based on publicly available mRNA sequences (Sargent et al. 2007). Novel markers referred to as Gene Pair Haplotype (GPH) markers are being developed in strawberry (Tom Davis personal communication) and are expected to be highly transferable from *F. vesca* to other strawberry species and even other genera in the Rosaceae. Many research groups are developing additional markers from the increasing sequence data available for strawberry and adding them to their diploid and octoploid linkage maps. The addition of codominant SSR, STS, gene-specific markers to these maps allows comparison among diploid and octoploid maps (Spigler et al. 2008, 2010) and assessment of colinearity among the homologous chromosomes and processes involved in the evolution of octoploidy in strawberry. Thermal asymmetric interlaced PCR (hiTAIL-PCR) was recently used to amplify the flanking region surrounding the left or right border of the T-DNA in 108 of these unique single copy mutants. Markers (based on presence/absence, length and CAPS polymorphism) were developed to 74 of the T-DNA insertion lines and were mapped in the reference diploid *F. vesca* 815 × *F. bucharica* 601 population (Ruiz-Rojas et al. 2010).

Efficient transformation protocols and availability of mutants are necessary for forward and reverse approaches of elucidating gene function. Several reviews on tissue culture and transformation of strawberry were published (Folta and Davis 2006; Debnath and da Silva 2007). Efficient *Agrobacterium*-mediated transformation and rapid regeneration appears genotype-specific in strawberry and has been reported for *F. vesca* "Hawaii-4" (Oosumi et al. 2006), *F.* ×*ananassa* "L-9" (Folta and Davis 2006). One approach, T-DNA mutagenesis or "gene tagging", to generate mutants is a technique used for generating loss-of-function mutations in genes by mobile or introduced DNA with a known sequence (T-DNA in this case) and was used in strawberry (Shulaev et al. 2008). These T-DNA mutants are expected to provide resources for reverse genetics in addition to novel markers as demonstrated by Ruiz-Rojas et al. (2010).

A comprehensive review of functional molecular and biotechnology studies in strawberry was recently published (Schwab et al. 2009).

In this genomic era, strawberry resources are expected to increase dramatically with increased federal funding and recent advances in next-generation sequencing. A Strawberry Genome Sequencing Consortium, comprised of experts in a wide array of research areas, was created in the spring of 2008 with the goal of sequencing the genome of "Hawaii-4" using next-generation technologies (Shulaev et al. 2010). Current support for GDR by the USDA Specialty Crop Research Initiative as part of tree fruit Genome Database Resources (tfGDR) will allow expansion of this database to include whole genome sequences and annotations for strawberry, transcript data, metacyc pathways, large-scale phenotype and genotype data, breeding data, controlled vocabularies, and new analysis tools. SCRI funding for "RosBREED: Enabling Marker-Assisted Breeding in Rosaceae" promises to deliver high-throughput genome scan platforms and integrate breeding and genomic resources by implementing marker-assisted breeding primarily in four fruit crops including strawberry (Iezzoni et al. 2010). The genome

sequence of *F. vesca* and bioinformatics tools to analyze such data through the GDR database, among others, will provide a valuable resource for future studies of comparative genomics in the Rosaceae, evolution of polyploidy in *Fragaria* and phylogenetic relationships among members of this economically important family of temperate fruits.

2.7 Functional Improvements

The strawberry fruit contains thousands of metabolites, which strongly impact consumer's senses and health (Schwab et al. 2009). Most analytical biochemical studies of strawberry fruits have relied on specific extraction/separation methods to identify and quantify specific compounds and interests. The strawberry flavor is complex. One comprehensive non-targeted metabolic analysis of strawberry identified 5,844 unique spectrophotometric peaks by analyzing fruits at four developmental stages (Aharoni et al. 2002). Many artificial strawberry flavors use only a handful of the top compounds to cheaply imitate the true constituents, and the human taste recognizes the difference. Schwab et al. (2009) summarizes the genetic work concerning volatile and polyphenolic compounds including metabolic routes and associated genetic mechanisms.

Fruit firmness, a genetically complex trait, has been a focal point of many large breeding programs during the past 50 years. Though "firm" strawberries is the primary complaint of consumers of commercial strawberry fruit throughout the world, this trait has provided the strawberry industry with the capability to move fruit to the far reaches of the globe and capitalize on strawberry as a product. Breeding for firmness is a difficult task, complicated as Salentjn et al. (2003) has pointed out, because of the inverse correlation between firmness and flavor emissions. Recent breakthrough in developing fruit with flavor and firmness are the new dictum of the present commercial breeding programs.

Strawberries are rich in vitamin C, ascorbic acid, and ellagic acid. Both compounds have a significant role in promoting human health. The amount of ellagic acid varies between cultivars and between different plant parts. Because of the variability of these compounds between different cultivars, molecular genetic studies will be examining major qualitative trait loci involved in strawberry vitamin C and ellagic acid biosynthesis to be mapped for molecular breeding efforts.

2.7.1 Allergens

As in other fruits, strawberries contain proteins, which can cause allergic reaction in humans (Schwab et al. 2009). The strawberry FRA a 1 protein family is homologous to the major birch pollen allergene Bet v 1 and includes several IgE-binding peptides with small intra- and intergenotype sequence variability though subjected to post-translational modifications.

Profilins and lipid transfer proteins (LTP), found in strawberries, are also represented in other cultivated crops in the rose family. Strawberry LTP and profilins are expressed in many fruit tissues and accumulate with abiotic stress (Yubero-Serrano et al. 2003). Some studies have found that strawberry LPT had lower allergenicity than apple or peach homologs. The strawberry allergens are in the range suited for immunotherapy (Zuidmeer et al. 2006).

2.8 Biotechnological Approaches to Strawberry Improvement: Benefits and Risks

2.8.1 Benefits

The potential for positive application of biotechnology to strawberry, as with other fruits and vegetables, is limited by the lack of public approval of breeding through genetic manipulation (Hummer and Hancock 2009; Mezzetti 2009). The cost of research and development is high, and regulatory approval is tortuous and prohibitive. Experimentation with perennials is expensive, relative to annual crops. Thus, biotechnological application of molecular and genetic development of fruit crops through transgenes has not progressed since the early 1980s, when techniques first became available. Transformation of the octoploid strawberry has been well-documented (Mezzetti 2009), but acceptance of the products has not been given, so the industry has suppressed this research.

If transgenes were accepted for strawberry development, many advances could be made efficiently including:

- Development of glyphosate resistant cropping systems, which could help farmers who have lost methyl bromide
- Improved root rot resistance – also help for the loss of methyl bromide
- Promoted flowering and fruiting
- Quality – maturation genes for a non-climacteric fruit
- Tissue softening genes (for firmness)
- Carbohydrate development for flavor and processing quality
- Disease and pest – virus diseases
- Cold hardiness
- Parthenocarpic fruiting gene

2.8.2 Risks

Several obstacles work against the acceptance of transgenic strawberries. The global economic value of this fruit crop (while high per acre) is small in total because much fewer acres are planted than that of agronomic crops. As a result, governments are not flocking to support this technology, and private stimulus is modest. The fruit industry has been reluctant to introduce products with potential negative backlash from people leery of consuming transgenic crops.

A second obstacle is the tendency of strawberries to be outcrossing. Their flower is open and insect pollination is the norm. In each of the locations, where strawberries are cultivated, native relatives are widespread. These species relatives could incorporate transgenes into wild biological systems. For this reason, release of transgenic strawberries will require more scrutiny and in depth ecological surveys than have been performed in other agricultural crops.

A strong influx of funds for thorough testing and environmental examination is needed before transgenic strawberries could be examined. Careful analysis of people's perceptions regarding transgenic fruit is also required. Until this happens, transgenic strawberries will remain as a research tool without commercialization. Using marker-free transformation systems and targeted expression of transgenes will minimize public concern, but the fear of technology must be abated before transgenic strawberries will be commonly accepted.

2.9 Recommendations for Future Actions

The studies of genetics and genomics of the *Fragaria* genome are proceeding at unparalleled rates. Comparative genomics and fine mapping can elucidate the processes involved in polyploidization and the evolution of sex determination in the octoploid species and are under way. Strawberry researchers are working within the rose family community and beyond to share information and relate genes and gene patterns. This newfound knowledge will be used by traditional breeders to develop improved cultivars in advanced fruit quality, expanded growing ranges, and during all seasons.

Recent findings have overturned some older paradigms. Previously, Staudt (1999a, b) suggested that the diploid *F. vesca*, an old species, could have an origin as early as the Cretaceous period. A significant preliminary finding using Bayesian analyses of complete chloroplasts obtained by high-throughput sequencing (Njuguna 2010a, b) contradicts this timeline and indicates that *Fragaria*, as a genus, is young and evolved less than 5 million years ago. Also, the octoploids evolved perhaps only 2.7 million years ago. Further exploration and study of *Fragaria* crop wild relatives using next-generation and even third generation technology will shed light on the evolution of *Fragaria* and its polyploidy. Exploration has confirmed that hybridization of strawberry species in nature, such as the production of *F.* ×*bringhurstii* and an unnamed Chinese pentaploid (Lei et al. 2005), and decaploid *F. virginiana* subsp. *platypetala* (Davis et al. 2010) is a more frequent occurrence than suggested by Darrow (1966) or Staudt (1999a, b).

Staudt (1999a, b) postulated that the first octoploid probably arose in East Asia and migrated from the west via an Alaskan–Siberian land bridge to North America. He had thought that *F. iturupensis*, which he first observed as the only Asian octoploid, might be a missing link. Decaploidy in *F. iturupensis* complicates this view. Further study of strawberries of northern Pacific Islands is needed to determine where other higher ploidy

strawberry colonies exist and what their phylogenetic role may have been. With the finding of the clustering of *F. vesca* subsp. *bracteata* with the North American octoploid species, the possibility of an American origin for the octoploids has been suggested. Additional explorations should be taken in Asia and North America to seek potential missing links that may have contributed to the evolution of the American octoploids.

This is a key time in uncovering the evolution of *Fragaria* and the development of the cultivated strawberry. Global interest and communication have brought the international strawberry research community together. The formation of a strawberry sequencing consortium and federal funding for many Rosaceae projects that include strawberry will lead to unprecedented discoveries for this model perennial crop. The International Treaty on Plant Genetic Resources recognized the importance of strawberry as an Annex 1 crop. The Global Crop Diversity Trust was instrumental in bringing together a scientific team to prepare a global conservation strategy for strawberry, which was completed (Hummer 2008, 2009). These activities have expanded the awareness of crop wild relatives for *Fragaria* in a positive fashion in most countries.

Yet the strawberry conservation strategy is not being implemented due to lack of resources. Several countries, where centers for diversity of strawberry species reside, have not recognized *Fragaria* as a sufficiently important genus worth establishing in a national genebank. Although individual universities, institutes, and scientists continue to study the genus and provide information, the security of wild strawberry species and landraces within these countries is unrecognized and potentially vulnerable to loss. Thankfully, the conservation strategy has been formed. Local implementation, institutional support, and world recognition is paramount for the continued conservation of critical landraces and subspecies and crop wild relatives of *Fragaria*.

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