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Chloroplast DNA Restriction Fragment Variation among Strawberry (*Fragaria* spp.) Taxa

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Abstract. **Restriction fragment-length polymorphisms (RFLPs) of chloroplast DNA (cpDNA) were used to study phylogenetic relationships among twenty-six *Fragaria* taxa and two closely related species, *Potentilla fruticosa* L. and *Duchesnea indica* (Andrews) Focke. Sixteen restriction enzymes and probes of the entire *Nicotiana tabacum* L. chloroplast genome revealed a very low level of variation among the *Fragaria* taxa, limiting phylogenetic resolution. However, *Fragaria* appears to be more closely related to *Potentilla* than *Duchesnea*. The diploid taxa, *F. iinumae* Makino, *F. nilgerrensis* Schlect. and *F. vesca* L. were the most divergent *Fragaria* taxa and *F. iinumae* appears to be the most ancestral taxon. Little variation was revealed within the economically important octoploid group of taxa, which gave rise to the cultivated strawberry, and no progenitor taxa to the octoploid group could be identified. The lack of variation in the chloroplast genome suggests that these *Fragaria* species may be of relatively recent evolutionary origin.**

Fragaria spp. (Rosaceae, Rosoideae, Potentilleae) are low-growing herbs endemic to various temperate environments in the northern and southern hemispheres. Morgan et al. (1994) studied sequence data of the chloroplast gene encoding the large subunit of ribulose-bisphosphate carboxylase (*rbc*L) to resolve many phylogenetic relationships between *Fragaria* and its closest relatives in Rosaceae, but phylogenetic relationships within the genus remain unclear. Current *Fragaria* taxonomy, based on cytology, interspecific fertility, and morphological information describes at least 16 *Fragaria* species with many subspecies (Table 1). Four basic fertility groups in the genus are associated primarily at the 2x, 4x, 6x, and 8x ploidy levels (Table 1) (Hancock and Luby, 1993). Species are further differentiated by morphological characters such as, leaf, flower, fruit, and runner traits, but these data provide only limited insight into the phylogenetic relationships among *Fragaria* taxa.

Table 1. Name, ploidy level and location of wild strawberry (*Fragaria*) species[z].

Species	Ploidy	Range
F. vesca L.	2x	North America, northern Asia, and Europe
F. viridis Duch.	2x	Europe and western Asia
F. nilgerrensis Schlect.	2x	Southern and eastern Asia
F. daltoniana J. Gay	2x	Himalayas
F. nubicola Lindl.	2x	Himalayas
F. iinumae Makino.	2x	Japan
F. yezoensis Hara.	2x	Japan
F. mandshurica Staudt	2x	Northern Asia
F. nipponica Lindl.	2x	Japan
F. corymbosa Losinsk	4x	Northern China
F. orientalis Losinsk	4x	Northern Asia
F. moupinensis French.	4x	Southern China
F. moschata Duch.	6x	Northern and central Europe
F. chiloensis (L.) Duch.	8x	North and South America, Hawaii
F. virginiana Duch.	8x	North America
F. iturupensis Staudt	8x	Japan
F. ×*ananassa* Duch.	8x	Cultivated form[y]

[z]Adapted from Hancock and Luby (1993).
[y]Interspecific complex of *F. chiloensis* and *F. virginiana*. Natural hybrids between *F. chiloensis* and *F. virginiana* are also endemic to parts of the Pacific Northwest (Staudt, 1962).

Genetic relationships have been studied among some *Fragaria* species in the diploid *F. vesca* and octoploid *F. chiloensis* (L.)

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Duch. and *F. virginiana* L., with studies of photosynthetic patterns (Cameron and Hartley, 1990; Chabot, 1978; Hancock et al., 1989; Jurik, 1979), mating systems, isozyme variation (Arulsekar and Bringhurst, 1981; Hancock and Bringhurst, 1978, 1979, 1980), and ecological and morphological variation (Angevine, 1983; Jensen and Hancock, 1981; Stahler, 1990). Few studies have included Eurasian taxa and none have placed this information into a phylogenetic context.

Many *Fragaria* species have desirable fruit, but only *F.* ×*ananassa* Duch., an interspecific-hybrid complex between the two octoploid species, *F. virginiana* and *F. chiloensis*, is commercially cultivated to any great extent. Although this hybrid occurs naturally in some parts of the Pacific Northwest of the United States and British Columbia (Staudt, 1989), the cultivated strawberry is essentially derived from artificial hybridizations. Compared to many cultivated crops, this origin is relatively recent, dating to the 18th century (Darrow, 1966), and years of selection for adaptation to cultivated environments has reduced genetic diversity within the cultivated strawberry (Dale and Sjulin, 1990; Luby et al., 1991; Sjulin and Dale, 1987). Because a narrow genetic base can be associated with inbreeding depression and vulnerability to diseases, pests, and environmental stresses (Luby and Stahler, 1993), breeders are interested in expanding the narrow genetic base of the strawberry by incorporating wild relatives into the breeding programs. Although many useful traits will come from the founding species, *F. chiloensis* and *F. virginiana,* beneficial genes may also come from closely related species. An understanding of the phylogenetic relationships among *Fragaria* species allows breeders to search the genus systematically based on genetic similarities to *F.* ×*ananassa*. These phylogenetic relationships may also reveal relationships with previously disregarded taxa that could lead to new sources of genes for genetic improvement.

Analysis of DNA restriction fragment-length polymorphisms (RFLPs) in the chloroplast (cpDNA) and nuclear genomes has provided information on phylogenetic relationships within many taxonomic groups, including *Brassica* (Palmer et al., 1983), *Zea* (Doebley et al., 1987), *Pennisetum* (Gepts and Clegg, 1989), *Persea* (Furnier et al., 1990), *Glycine* (Doyle et al., 1990), and *Vitis* (Bourquin et al., 1993). Analysis of cpDNA has been particularly informative because the genome is small, simplifying interpretation, and the rate of evolution is slow enough that simple variation can be detected among genera, species, and occasionally within species (Palmer 1986). Chloroplast DNA is also unaffected by changes in ploidy that can complicate phylogenetic analyses (Palmer 1986), a factor particularly important in *Fragaria*, with its array of ploidy levels. In most angiosperms, cpDNA is inherited maternally, although biparental (Smith et al., 1986) and paternal inheritance (Schumann and Hancock, 1989) have also been reported. We used cpDNA RFLPs to estimate phylogenetic relationships among a sample of *Fragaria* taxa.

Table 2. Geographic origins and taxa information of the accessions.

Taxa		Ploidy	Origin	Source/accession no.
1	*Fragaria vesca* ssp. *californica*	2x	California	NCGR[z]/FRA 371
2	*F. vesca* ssp. *americana*	2x	Nebraska	Univ. of MN/N92143
3	*F. vesca* ssp. *vesca*	2x	Finland	NCGR/FRA 438
4	*F. vesca* ssp. *vesca*	2x	Sweden	NCGR/FRA 510
5	*F. viridis*	2x	Germany	NCGR/FRA 333
6	*F. viridis*	2x	Germany	NCGR/FRA 341
7	*F. nilgerrensis*	2x	China	Berlin Bot. Garden/053-10-84-40
8	*F. nilgerrensis*	2x	Yunnan Provice, China	Berkely Bot. Garden/84.0771[y]
9	*F. iinumae*	2x	Japan	NCGR/FRA 377
10	*F. iinumae*	2x	Japan	Univ. of California, Davis[x]
11	*F. nubicola*	2x	Pakistan	NCGR/FRA 520
12	*F. orientalis*	4x	Asia	NCGR/FRA 536[w]
13	*F. moschata*	6x	Germany	NCGR/FRA 609
14	*F. moschata*	6x	Europe	NCGR / FRA 157
15	*F. virginiana* spp. *virginiana*	8x	Minnesota	Univ. of MN/N92145
16	*F. virginiana* spp. *virginiana*	8x	Michigan	Univ. of MN/N92144
17	*F. virginiana* spp. *virginiana*	8x	New Hampshire	NCGR/FRA 381
18	*F. virginiana* ssp. *virginiana*	8x	Maryland	NCGR/FRA 67
19	*F. virginiana* ssp. *platypetala*	8x	Nevada	NCGR/FRA 370
20	*F. virginiana* ssp. *platypetala*	8x	Oregon	NCGR/FRA 99
21	*F. virginiana* ssp. *glauca*	8x	Wyoming	NCGR/FRA 104
22	*F. chiloensis* ssp. *pacifica*	8x	Alaska	NCGR/FRA 606
23	*F. chiloensis* ssp. *lucida*	8x	California	NCGR/FRA 366
24	*F. chiloensis* ssp. *chiloensis*	8x	Chile	NCGR/FRA 393
25	*F. chiloensis* ssp. *chiloensis*	8x	Peru	NCGR/FRA 372
26	*F. chiloensis* ssp. *chiloensis*	8x	Colombia	NCGR/FRA 24
27	*Potentilla fruticosa* 'Jackmans'	2x	Cultivar	Willowood Gardens; Bowler, Wis.
28	*Duchesnea indica*	12x	Southern and eastern Asia	North. Hort. Soc./490[v]

[z]National Clonal Germplasm Repository, Corvallis Ore.
[y]Originally classified as *F. moupinensis* by BBG; Reclassified by NCGR and G. Staudt (K. Hummer, curator at NCGR, personal communication).
[x]Received from R.S. Bringhurst, specific origins unknown.
[w]Now classified as "F. hybrid" by NCGR due to questionable pedigree (K. Hummer, curator at NCGR, personal communication; also see text).
[v]Seed lot from the Northern Horticultural Society; Harrogate, England; 1991 catalog no. 490.

Table 3. Probes derived from the SolClone Top40 clone bank[z] of *Nicotiana tabacum* cpDNA.

Probe	Size (kb)	Coordinates[y]	Subclones[x]
SC1-8	23.4	153746–21947	1–8
SC9a-15	21.5	21947–47699	9a,9b,10–15
SC16-24	26.4	47699–74167	16–19,20a,20b,21–24
SC25-33	27.0	74167–104801	25–28,29a,29b,30,33
SC34-40	25.8	104801–130600	34–40

[z]Olmstead and Palmer (1992).
[y]Coordinates for the *Nicotiana tabacum* cpDNA sequence (Shinozaki et al., 1986).
[x]Original subclone reference numbers for SolClone Top40.

Materials and Methods

We assayed 26 *Fragaria* accessions representing 16 different taxa from 9 species (Table 2). *Potentilla fruticosa* and *Duchesnea indica* are closely related to *Fragaria,* and these accessions were included as comparative outgroup species (Darrow, 1966; Morgan et al., 1994). Plants were rooted in 15-cm pots in a 2 peat : 1 sand : 1 perlite (by volume) medium from runners or divisions of the original plant during Summer 1992. They were first grown under greenhouse conditions and then moved to a cold frame in July. In early November the plants were moved to a 4 °C cooler for 8 weeks and in early January 1993 they were returned to the greenhouse.

In February, about 1 g of newly expanded or expanding leaf tissue was collected from each accession and used to isolate total cellular DNA (Doyle and Doyle, 1990). DNA yields using this method averaged 0.1 mg DNA/g of fresh tissue. One microgram of DNA per sample was digested separately with 10 units of the restriction enzymes, *BamH*I, *Bgl*I, *Bgl*II, *Bst*XI, *Cfo*I, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*II, *Kpn*I, *Msp*I, *Sal*I, *Sst*I, and *Xba*I. DNA fragments were separated by electrophoresis on 0.8% agarose gels in 1× TBE buffer (0.09 M tris-borate and 0.002 M EDTA; Sambrook et al., 1989). DNA was transferred to nylon membranes (Magnagraph; Micron Separations, Inc., Westboro, Mass.) using 20× SSC (3 M NaCl and 0.3 M sodium citrate; Sambrook et al., 1989) as a transfer buffer. DNA fragments were hybridized with ^{32}P-labelled (Feinberg and Vogelstein, 1984) or digoxigenin-labelled (Boehringer Mannheim Corp.; Indianapolis) *Nicotiana tabacum* chloroplast DNA (cpDNA) fragments (Olmstead and Palmer, 1992). Adjacent fragments were combined into five composite probes ranging in size from 23.4 to 27.0 kb, which together covered the entire chloroplast genome of *N. tabacum* (Table 3). Hybridizations and washes of the membranes followed the methods of Palmer (1986), and fragments were visualized by autoradiography or chemiluminescence. Hybridized probe was removed before rehybridization in washes of 0.4 M NaOH at 42 °C for 30 min, followed by 30 min at 42 °C in 0.1× SSC, 0.5% SDS (sodium dodecyl sulfate), and 0.2 M tris-HCl (pH 7.5).

When we could determine that a polymorphism was due to a particular restriction site mutation, we used it as a single character in the analysis. However, many polymorphisms could not be explained as simple restriction site changes. These polymorphisms may be due to more complex structural rearrangement differences between the taxa. As a conservative approach, if a set of polymorphic fragments was common among a group of taxa, these were interpreted as a single character (e.g., mutation 5, Table 4). However, each combination of restriction enzyme and probe was scored independently, meaning that some mutations could have been be scored more than once. We estimated phylogenetic relationships by parsimony analysis, using the bootstrap method with heuristic search in PAUP 3.0q (Swofford, 1990). A bootstrap 70% majority-rule consensus tree was developed using 100 replications.

Results and Discussion

Of 127 mutations observed, 61 involved only the outgroup species *P. fruticosa* and *D. indica*, leaving 66 phylogenetically informative mutations for analysis (Table 4). Fifteen of the observed mutations could be classified as restriction site changes (e.g., mutation 1, Table 4). Structural rearrangements between the chloroplast genomes of *Nicotiana* and *Fragaria* and *Potentilla* and *Duchesnea* have apparently been sufficiently numerous and complex to prevent us from discerning the nature of the remaining mutations (Table 4).

Variation within *Fragaria* proved to be low, limiting the resolution of the phylogenetic analysis. Several relationships, however, were resolved (Fig. 1). *Fragaria* and *Potentilla* appeared to be more closely related than *Fragaria* and *Duchesnea*, sharing six characters (mutations 4, 9, 13, 14, 21, and 35, Table 4) vs. only one (mutation 10, Table 4). This relationship is supported by fertility data, in which hybrid seedlings have been obtained from *F.* ×*ananassa* x *P. fruticosa* crosses, but not from *D. indica* x *F.* ×*ananassa* (Rose et al., 1993).

The diploid taxa, *F. iinumae*, *F. nilgerrensis*, and the *F. vesca* ssp. *vesca,* were the most divergent of the *Fragaria* taxa (Table 4, Fig. 1). *Fragaria iinumae* was separated from the other taxa by 21 characters (Fig. 1) and shared more characters with *Potentilla* than any other *Fragaria* taxon (mutations 4, 13, 21, 35, Table 4), suggesting that it is the most ancestral of the *Fragaria* taxa studied. It is also morphologically distinct, being the only deciduous species in the genus and the only diploid species with glaucous leaves (Luby et al., 1991; Staudt 1989).

Similarly, *F. nilgerrensis* is divergent, separated from the other *Fragaria* taxa by 11 characters (Table 4, Fig. 1). It is also genetically quite distinct, based on interspecific sterility (Dowrick and Williams, 1959) and morphological characteristics (Staudt, 1989). Eight characters separate the European *F. vesca* ssp. *vesca* from all other taxa, suggesting that this taxon is relatively divergent from most other *Fragaria* taxa, but partial fertility has been reported among *F. vesca*, *F. viridis* Duch., *F. nubicola* Lindl., and *F. nilgerrensis* (Dowrick and Williams, 1959; Hancock et al., 1991).

Characters shared by *F. orientalis* Losinsk (2n = 4x = 28) and *F. moschata* Duch. (2n = 6x = 42) suggest that these species may represent a polyploid series (Fig. 1). However, visual inspection of the *F. orientalis* accession (USDA accession FRA 536) suggests that there is some question concerning the assignment of this particular accession to *F. orientalis* (Table 2). This accession presumably derives from open pollination and may not accurately represent pure *F. orientalis*, so the USDA National Clonal Germplasm Repository is now classifying it as "F. hybrid". If the maternal plant was correctly labelled and the cpDNA was inherited maternally, then our data should accurately represent the cpDNA of *F. orientalis*. Chromosome counts of this accession were 28, as

Table 4. Descriptions of phylogenetically informative mutations observed among *Fragaria* taxa and between *Fragaria* taxa and the outgroup species.

Mutation[z]	Enzyme	Probe	Polymorphic fragments		Taxa[y]
			Present (Kb)	Absent (Kb)	
1	*Bam*HI	SC1-8	1.2 + 0.8	2.0	5,6
2	*Bam*HI	SC1-8	12.8		15–26
3	*Cfo*I	SC1-8	4.3	1.85	7,8
4	*Cfo*I	SC1-8	1.6	1.85	9,10,27
5	*Cfo*I	SC1-8	9.0,1.6	2.9,1.6	9,10
6	*Cfo*I	SC1-8		2.0	9,10
7	*Cla*I	SC1-8	4.8		15–26
8	*Dra*I	SC1-8	0.7		7,8
9	*Eco*RI	SC1-8	9.1	4.2	7,8,27
10	*Eco*RI	SC1-8	3.6	2.9	9,10,28
11	*Eco*RI	SC1-8	3.0		9,10
12	*Hind*III	SC1-8	1.9		15–26
13	*Hind*III	SC1-8	2.8		9,10,27
14	*Msp*I	SC1-8	4.4	6.2	7,8,27
15	*Sal*I	SC1-8	11.4		9,10
16	*Xba*I	SC1-8	2.3 + 2.1	4.4	9,10
17	*Xba*I	SC1-8	3.7	2.7 + 1.0	3
18	*Xba*I	SC1-8	2.4	1.3 + 1.1	7,8
19	*Xba*I	SC1-8	3.2		9,10
20	*Xba*I	SC1-8	2.0		15–26
21	*Bam*HI	SC9a-15	7.7 + 1.2	9.9	9,10,27
22	*Bgl*II	SC9a-15	0.9		7,8
23	*Cfo*I	SC9a-15	3.3	1.8 + 1.5	7,8
24	*Cla*I	SC9a-15	6.4	7.2	9,10
25	*Dra*I	SC9a-15	8.9	5.6 + 3.3	9,10
26	*Dra*I	SC9a-15	3.9	4.1	5–8
27	*Dra*I	SC9a-15	1.0	2.6	3,4
28	*Eco*RI	SC9a-15	1.4 + 0.5	1.9	7–8
29	*Eco*RV	SC9a-15	4.3,3.1	5.9	3,4
30	*Eco*RV	SC9a-15	2.4		9,10,15–26
31	*Hind*III	SC9a-15	1.9 + 0.9	2.8	9,10
32	*Hpa*II	SC9a-15	3.3	1.7 + 1.6	7,8
33	*Kpn*I	SC9a-15	4.7	5.3	9,10
34	*Sal*I	SC9a-15	9.7		9,10
35	*Bam*HI	SC16-24	5.9 + 1.0	6.9	9,10,27
36	*Bst*XI	SC16-24	10.6,8.8		5–10
37	*Cfo*I	SC16-24	4.0	3.7	5,6,9,10,15–26
38	*Cla*I	SC16-24	4.0	4.7	3,4
39	*Cla*I	SC16-24	4.5	4.7	7,8
40	*Dra*I	SC16-24	4.3 + 0.3	4.6	9,10
41	*Dra*I	SC16-24	2.6	3.0	12–14
42	*Dra*I	SC16-24	1.3		9,10
43	*Dra*I	SC16-24		1.5	1–4,9,10
44	*Eco*RI	SC16-24	1.4 + 0.5	1.9	7,8
45	*Hind*III	SC16-24	0.9	2.9	9,10
46	*Dra*I	SC25-33	1.3 + 1.1	1.4	13,14
47	*Dra*I	SC25-33	4.1	4.7	9,10
48	*Eco*RI	SC25-33	11.6		9,10
49	*Eco*RI	SC25-33	1.1		7,8
50	*Hind*III	SC25-33	6.0		9,10
51	*Kpn*I	SC25-33	8.4,10.0		9,10
52	*Bgl*I	SC34-40	2.8		3,4
53	*Bgl*II	SC34-40	6.9		9,10
54	*Bst*XI	SC34-40	9.4		3,4
55	*Bst*XI	SC34-40	12.3		9,10
56	*Bst*XI	SC34-40	12.3	13.3,20.0	12–14
57	*Kpn*I	SC34-40	7.2		3,4
58	*Kpn*I	SC34-40	7.4		7,8
59	*Eco*RI	SC34-40	3.7	3.0 + 0.7	12–14
60	*Eco*RI	SC34-40	5.4		1,3,4
61	*Eco*RV	SC34-40	7.5		4
62	*Kpn*I	SC34-40	1.6,1.2		3,4
63	*Kpn*I	SC34-40	8.4		9,10
64	*Sst*I	SC34-40	5.6		3,4
65	*Sst*I	SC34-40	4.2		18,19
66	*Xba*I	SC34-40	8.2		3

[z]All mutations within the large single copy region of the chloroplast genome.
[y]Numbers of taxa are as given in Table 2.

expected for *F. orientalis* (Staudt, 1989), but data from an accurately classified accession of *F. orientalis* are necessary to clarify this situation.

Although the octoploid taxa have considerable geographical, morphological, and allozyme variation (Hancock and Bringhurst, 1981), they display strikingly little cpDNA variability, with all taxa grouped together by six mutations (mutations 2, 7, 12, 20, 29, 37, Table 4). No species were clearly identified as ancestral to the octoploid taxa even though *F. vesca* has been implicated using cytogenetic information (Bringhurst and Khan, 1963; Senanayake and Bringhurst, 1967). Interspecific fertility among octoploids is high (Hancock et al., 1991), also suggesting that these species are closely related and may share a common ancestral maternal origin.

The value of cpDNA polymorphisms in establishing phylogenetic relationships is well documented (Palmer, 1987); however, sequence variability can be low in some cases, limiting phylogenetic resolution (Olmstead et al., 1990; Rieseberg et al., 1991). Schilling and Jansen (1989) observed no variation among a group of geographically and morphologically diverse *Viguiera* taxa and concluded that these species were of recent divergence from the other closely related genera in the study. In general, the *Fragaria* taxa studied had lower levels of mutation as ploidy levels increased, and this may suggest that the higher ploidy levels, including the economically important octoploid taxa, are of relatively recent origin as compared with the diploid taxa.

The lack of variation among the octoploid taxa may suggest that these taxa are derived from a common ancestor. The precise origin of the octoploids remains unknown; however, the range of *F. chiloensis* extends west along the Aleutian Islands and another octoploid species, *F. iturupensis* Staudt, occurs in the Kuril Islands (Staudt, 1973), suggesting that polyploidization could have occurred in eastern Asia with the octoploid taxa then spread across the Bering Strait land bridge into North America.

The highest levels of polymorphism were among *Fragaria*, *Potentilla*, and *Duchesnea*, suggesting that cpDNA RFLP analysis is better suited for assessing variation at the subfamily (Rosoideae) and tribe (Potentilleae) levels and could potentially resolve some of the remaining questions concerning the ancestry of many of the economically valuable genera within the Rosaceae family.

Future phylogenetic studies of *Fragaria* will likely include Mendelian markers to reveal more variability among the taxa and, in conjunction with these cpDNA data, can reveal cases of interspecific hybridization (Furnier et al., 1990). This molecular information with the vast amounts of morphological and cytological data previously compiled (Darrow, 1966; Staudt, 1962, 1989) will allow breeders to incorporate nondomesticated germplasm more effectively in breeding programs.

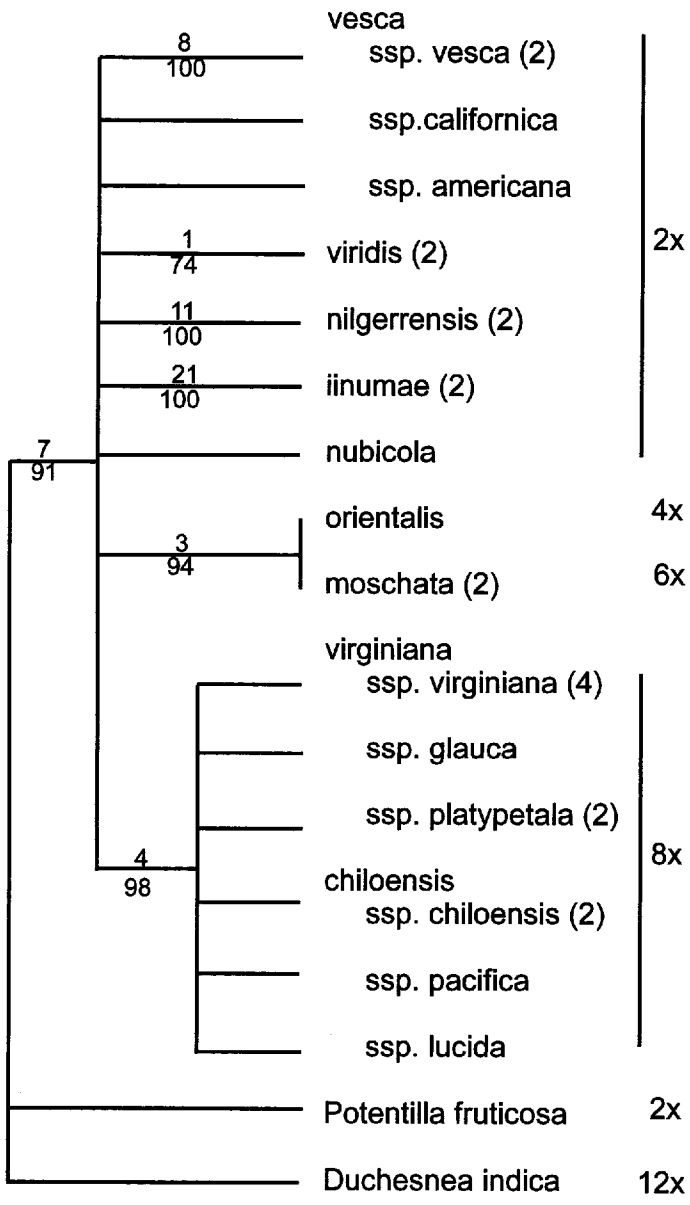

Fig. 1. Cladogram of the bootstrap 70% majority-rule consensus tree using 100 replications. The number of mutations that define each branch is shown above the branch lines and the number of times that each branch occurred out of 100 bootstrap replications is shown below the branch lines. Ploidy levels are shown at the right and the number of accessions representing a given taxa, if >1, are in parentheses.

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