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Intraspecific classification of *Fragaria chiloensis*: a molecular approach to germplasm protection and utilization.

by

Suzanna Porebski

Table of Contents: Page

Intraspecific classification of *Fragaria chiloensis*: a molecular approach to germplasm protection and utilization.

Sue Porebski
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Abstract:

Fragaria chiloensis is one of the progenitors of the most widely cultivated strawberry crop, *F.* X *ananassa* Duchesne. Four subspecies defined on a geographic basis have been proposed, but never supported with quantitative data; ssp. *lucida* (E. Vilm.) and ssp. *pacifica* Staudt from North America, ssp. *chiloensis* Duchesne from South America and ssp. *sandwicensis* (Decaisne) Staudt from the Hawaiian Islands. The main objective of the present research was to develop a classification that was both operational and an accurate portrayal of genetic relationships in order to achieve efficient protection and utilization of germplasm for use in crop improvement. A discriminant analysis based on 14 measurements in 95 herbaruim specimens revealed a distinct separation of the Hawaiian ssp. *sandwicensis* which were shown to have longer leaflets, longer hairs on the undersurface of the leaflets and more numerous leaflet veins. The South American and North American plants were significantly different but overlapped to a degree. The former differed primarily by having 6-10 petals, instead of having 5-6 (rarely 7) petals. The two North American subspecies overlapped extensively with the only morphological distinction being hair type. These relationships were then further investigated using a molecular approach. DNA was removed from fresh leaf tissue using a modification of a CTAB DNA extraction protocol for 54 plants including 5 referable to North American ssp. *lucida*, 15 referable to North American ssp. *pacifica*, 15 referable to South American ssp. *chiloensis* and 19 from ssp. *sandwicensis* and were analysed using random amplified polymorphic DNA (RAPDs). From 100 primers screened, 12 were selected providing 68

scorable polymorphic bands. The phenogram (cophenetic correlation, r=0.97) based on UPGMA clustering of Jaccard's coefficients revealed a clear division between North American and South American plants but only partial separation between the two North American subspecies thus, generally supporting the classification based on morphology. Due to relatively limited genetic separation, extensive geographic overlap and minimal morphological variation, it is proposed that the two North American subspecies be reduced to the rank of forma: Since the name *lucida* has priority over *pacifica*, the resulting taxonomic change creates *Fragaria chiloensis* (L.) Duchesne ssp. *lucida* (E. Vilm) f. *lucida* and f. *pacifica* (Staudt) Catling & Porebski. Since the North American ssp. *lucida* and Hawaiian ssp. *sandwicensis* are genetically distinct, they provide a valuable source of novel genes for incorporation into the crop. The substantial effort of the Canadian Clonal Genebank to protect native Canadian wild *Fragaria chiloensis* germplasm is thus supported.

Key Words: strawberry, *Fragaria chiloensis*, ssp. *chiloensis*, ssp. *pacifica*, ssp. *lucida*, ssp *sandwicensis*, RAPD, morphology, geography, variation, classification, germplasm, Canada, United States, Chile, Hawaiian Islands.

La classification intraspécifique de *Fragaria chiloensis*: Une approche molécuaire à la protection et à l'utilisation des génes.

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Résumé:

Fragaria chiloensis est une espèce ascendante de *F.* X *ananassa* Duchesne, ce dernier étant le fraisier le plus cultivé. Ce groupe englobe présentement quatre sous-espèces qui sont déliniées sur une base géographique; ssp. *lucida* (E. Vilm.) et ssp. pacifica Staudt provenant de l'Amérique du Nord, ssp. *chiloensis* Duchesne provenant de l'Amérique du Sud, et ssp. *sandwicensis* (Deca.sne) Staudt provenant des Îles d'Hawaï. L'objectif principal de cette recherche était d'examiner les variations de l'espèce *F.chiloensis*, développer une classification fonctionnelle, démontrer avec précision les relations génétiques, ainsi permettre une protection efficace des variations génétiques et l'amélioration des cultures. Une analyse basé sur 14 measures de 95 spécimens d'herbier a permis de distingué l'espèce Hawaïenne ssp. *sandwicensis* des autres puisqu'elle avait un feuillage plus allongé, en dessous une pubesecence à poils plus longs et comprenait de nombreuses veinures. Les plantes de l'Amérique du Sud et du Nord étaient significativement différentes, mais avait aussi certains caractéristiques similaires. L'espèce du sud ce différenciait en ayant normalement 6-10 pétales tandis l'espèce du nord avait 5-6 et rarement 7 pétales. Les deux espèces du nord étaient très similaires à exception d'une différence morphologique dans leur du type de pubescence. Ces similairités ont été étudiées d'avantage en utilisant l'approche moléculaire d'ADN. Des tissues frais provenant du feuillage ont été prélevés de cinquante quatre plantes différentes en utilisant la technique d'extraction CTAB ADN, ensuite analysés en utilisant la technique d'ADN polymorphe amplifié au hasard (RAPDs). Ces espèces comprenaient 5 provenant de sous-espèces de

l'Amérique du Nord *lucida*, 15 de sous-espèces de l'Amérique du Nord *pacifica*, 15 de sous-espèces de l'Amérique du Sud *chiloensis* et 19 de sous-espèces des Îles de Hawaï *sandwicensis*. Des 100 amorces différentes, 12 ont produites des données formant 68 bandes de polymères mesurables. Le phénograme (cophenetique corrélation, r=0.97) basé sur les UPGMA grappe de Jaccard, les coéfficients démontrent une différence évidente entre les plantes provenant de l' Amérique du Nord et celles de l'Amérique du Sud, mais une différence mimime entre les deux sous-espèces de l'Amérique du Nord. Dû aux variations génétiques limitées, le chevauchement géographique et les variabilités morpholegiques, il est suggeré que les sous-espèces de l'Amérique du Nord soient modifiés à une forme. Puisque le nom *lucida* a priorité du nom *pacifica*, les changements taxonomiques donne comme résultats les sous-espèces suivantes; *Fragaria chiloensis* (L.) Duchesne ssp. *lucida* (E. Vilm) f. *lucida* et f. *pacifica* (Staudt) Catling & Porebski. Les distinctions et relations entre les sous-espèces de *F. chiloensis* démontrent que les sous-espèces de l'Amérique du Nord d'Hawaï peuvent être des sources utiles. En utilisant les variabilités génétiques on pourrait amélirorer les cultures et en plus, ces recherches appuient les efforts substantielles de la "Banque des gênes du Canada" qui vise à protèger les plantes indigènes canadiennes, incluant les fraisiers indigènes.

Mots clés: Fraises, *Fragaria chiloensis*, ssp. *chiloensis*, ssp. *pacifica*, ssp. *lucida*, ssp. *sandwicensis*, AND polymorphe amplifié au hasard (RAPD), morphologie, géographie, variation, classification, gêne, Canada, État-Unis, Chilie, Îles d'Hawaï.

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List of Tables: **Page**

List of Figures: **Page**

Introduction:

i). Needs:

Strawberries have been shown to have a very narrow genetic base (Luby et al. 1992), since most cultivars released since 1960 come from only a few founding ancestors, selected for specific characteristics (Dale & Sjulin 1990). A narrow genetic base can lead to "detrimental inbreeding effects" and loss of diversity which reduces tolerance to environmental fluctuations. To broaden the genetic base it is necessary to incorporate genetic traits from wild plants and the classification system provides the initial orientation to the genetic diversity of wild relatives (Hancock & Luby, 1993a, b). There has been a need for the systematic revision of *Fragaria* for a long period (Staudt 1989). This has become increasingly urgent over the past decade with attempts to protect native wild germplasm and develop crops with resistance to pests and pathogens (Luffman & MacDonald 1992, Cohen *et al.* 1991) as well as stress tolerance (Hancock & Luby 1993a, b).

From wild relatives, important germplasm can be obtained to help reduce chemical usage for disease and pest regulation and also increase production efficiency (Daubeny 1990). Disease and pests are often the limiting factors with regards to production and fruit quality (Galletta *et al.* 1989; Hancock *et al.* 1991). Traits have been identified in wild strawberries which are commercially beneficial and when incorporated into cultivated strawberries, broadening the genetic base, better adaptation to environmental fluctuations can result (Dale 1994). In the past, evaluation of the Californian *F.chiloensis* has lead to improved disease and pest resistance (Leppik 1970), to aphid black vine weevil (Crock et al. 1982), two spotted spider mite (Shanks & Barritt 1975), red stele rot and to verticillium wilt (Bringhurst *et al* 1966). The need for more information with regards to patterns of variations, both intra and interspecific, has been expressed (Williams 1993) along with

the need for improved taxonomy (Stalker 1989, Staudt 1989, Small & Catling 1995, 1996). Specifically, our general knowledge in Canada with regards to genetic variation is inadequate to deal with germplasm protection and crop improvement.

ii). Importance of Strawberries in Canada:

Next to apples, strawberries are the second most important clonal crop in Canada with an annual farmgate value exceeding 62 million dollars (Statistics Canada 1995, Davidson 1995, Moore *et al.* 1991). They account for 14.5% of the total recorded value of clonal crops (Small & Catling, 1995). In addition to crop value, present breeding requirements, danger of extinction and future crop value are all important when considering germplasm preservation (Small & Catling 1996). The relative value of wild germplasm can be asssesssed by examining the holdings in germplasm banks like the Canadian Clonal Genebank, Germplasm Resources Information Network and the United States Department of Agriculture. The North American clonal repositories recognize the substantial value of wild germplasm with the Canadian Clonal Genebank having 66% of wild material which is dominated by economically important genera (Small & Catling 1995). In fact, a very large percentage of the accessions of *Fragaria* in the Canadian Clonal Genebank are obtained from wild strawberries along the Pacific coast (Jamieson 1996). Not only is wild germplasm important but worldwide awareness and the rate of utilization of wild resources is also increasing (Moore & Ballington 1991, Brown 1989). Currently, strawberries have been considered a high priority with regards to germplasm preservation (Catling & Cayouette 1994, Catling & Porebski 1996, Catling 1996) and need for an accurate and informative clasification system (Staudt 1962, 1989, Bringhurst 1990).

iii). Contributions of Systematics:

Systematics is concerned with biological variation among living things and their evolution and may include phylogenetic analysis and also taxonomy, which deals with classification, names and identification (Small 1989, 1993, Systematic Agenda 2000 1994). The demands for systematics have been steadily increasing with the recognition that systematics is a "foundation" for all disciplines in biology (Small 1993) and management of world resources. With proper identification and a classification system organizing the genetic variability in wild relatives, wild resources can be effectively utilized to improve crops (Abelson 1991). Taxonomic support is very important to identify and preserve the range of diversity (Van Der Maesen 1990, Small 1993). In order to monitor biodiversity, systematics can help discover, describe and inventory species including any species in danger of extinction (Systematics Agenda 2000 1994) and aid in crop improvement. Much of the basic classification of native Canadian plants, especially strawberries and other berry plants, is inadequate and the extent of our understanding of their genetic variation is lacking (Catling & Porebski 1996). With a variety of techniques and especially molecular technology which has increased the power and accuracy of systematics significantly, access to the genes of all organisms, clarification of systematic affinities and clear phylogenetic relationships are now possible (Moritz & Hillis 1990).

iv). Practical Significance:

At present, there is inadequate information in Canada to successfully protect wild germplasm (Catling 1996) due to a lack of systematic knowledge and limited funding. We must insure that all

necessary germplasm is assessed so that we may establish and assign accurate priorization for germplasm protection and also to maintain options for crop improvement (Sjulin & Dale 1987, Luffman 1996, Jamieson 1996, Hodgkin *et al.* 1995). Canadian breeders have begun to exploit wild Canadian clones with beneficial results in eastern, western and central Canada (Catling 1996). Future success will depend on a continuing program of improvement utilizing accurately identified and characterized wild germplasm. Groups of berry plants in Canada, potentially strawberries, can be a world resource as far as the protection of genetic diversity of wild crop relatives is concerned. (Davidson 1995, California Gene Resource Program 1982). *Fragaria chiloensis* is one of the progenitors of the widely cultivated Dessert Strawberry (*F.* X *ananassa*) (Staudt 1989). Any increased knowledge indicating patterns of genetic variation can help us compensate for the narrow germplasm base of cultivated strawberries and, in effect, aid germplasm protection and utilization (Bringhurst *et al.* 1977) and crop improvement.

v). General Objectives:

The primary objective of this thesis is to examine the pattern of variation within *F.chiloensis* and to develop a classification that is both operational and an accurate portrayal of genetic relationships. Improvements in classification are anticipated to lead to a more efficient protection of genetic variation for use in crop improvement and for direct utilization based on an improved understanding of genetic relationships.

vi). Methodology:

Within the thesis, hypothesis refinement is achieved through examination of morphological

and geographical variation. Hypotheses were tested using statistical evaluation of random amplified polymorphic DNA (RAPDs). This technique uses one single short primer as opposed to a pair of primers. The primers anneal in specific places and serve as a starting and ending point for the Taq polymerase synthesis of a new DNA strand. Multiple bands are generated by having multiple annealing sites for a particular primer (Swenson 1997). This technique has proven to be successful in the past for similar taxonomic investigation of inter/intraspecific variation in *Juniperus*, *Salinornia*, *Marchantia*, *Asphodelus* and *Syringia* (Adams & Demeke 1992, Demeke *et al.* 1992, Marsolais *et al.* 1993, Parent *et al.* 1993, Boisselier-Dubayle *et al.* 1995, Luque et al. 1995, Lifante & Aquinagalde 1996).

Recently, RAPDs have been used to assess variation among octoploid strawberry cultivars (Hancock *et al.* 1994) and for determining variation within populations of octoploid species *F. virginiana* and *F. chiloensis* populations (Harrison *et al* 1994; Harrison *et al.* 1997). Although the lack of allelism may present a problem in RAPDs (Devos & Gale 1992) since it is impossible to confirm whether similar bands scored originate from the same allele site, the probability of similar origin is high in this study due to the low taxonomic level examined. Furthermore, polymorphism is maximized through adequate primer screening and selection to combat loss due to the lack of heterozygote detection through the dominance of banded over null alleles within polyploids. With continuous refinement of the RAPD technique and through the maintenance of stringent conditions, RAPDs have been shown to be a valuable tool for assessing genetic polymorphism (Swensen 1997; Welsh & McClelland 1990; Williams *et al.* 1990). This technique shows promise for detecting the pattern of variation present in wild strawberry populations (*in situ*) and also in collections stored in clonal genebanks (*ex situ*). Such a technique, in comparison to another technique like DNA

sequencing, provides an relatively inexpensive means of identifying valuable germplasm from that which is genetically redundant.

vii) Publications:

The work described here was initiated in partial response to requests for assistance from the AAFC clonal genebank and largely completed with resources provided by AAFC. The original plan included publication of results. At the time of submission, one paper was published on the protocol as a commentary in Plant Molecular Biology Reporter 15(1): 8-15, one was accepted on the morphological work in Can. J. Bot. and two others on the RAPD analyis were submitted for publication in Plant Genetic Newsletter and Can. J. Bot.

CHAPTER 1: Taxonomic Overview:

1.1). Classification of genus Fragaria:

Fragaria is classified according to Scoggan (1978) as follows:

CLASS.....DICOTYLEDONAE

FAMILY.....ROSACEAE

ORDER.....ROSALES

GROUP 1: (Chromosome number 7,8 or 9) 4 Subfamilies

1. Spiraeoideae.(Spiraeeae , Exochordeae, Holodisceae)
2. Neuradoideae (*Neurada*)
3. Prunoideae (*Prunus, Amygdalus, Armeniaca, Cerasus, Laurocerasus, Padus, Persica*)
4. Rosoideae

Tribe 1.....Ulmarieae (*Filipendula, Ulmaria*)

Tribe 2.....Kerrieae (*Kerria, Rhodotypos*)

Tribe 3.....Potentilleae (***Fragaria***, *Dryas, Geum, Potentilla, Rubus*)

Tribe 4.....Cercocarpeae (*Cercocarpus*)

Tribe 5.....Sanguisorbeae (*Acaena, Agrimonia, Alchemilla, Poterium, Sanguisorba*)

Tribe 6.....Roseae (*Hutthemia, Rosa*)

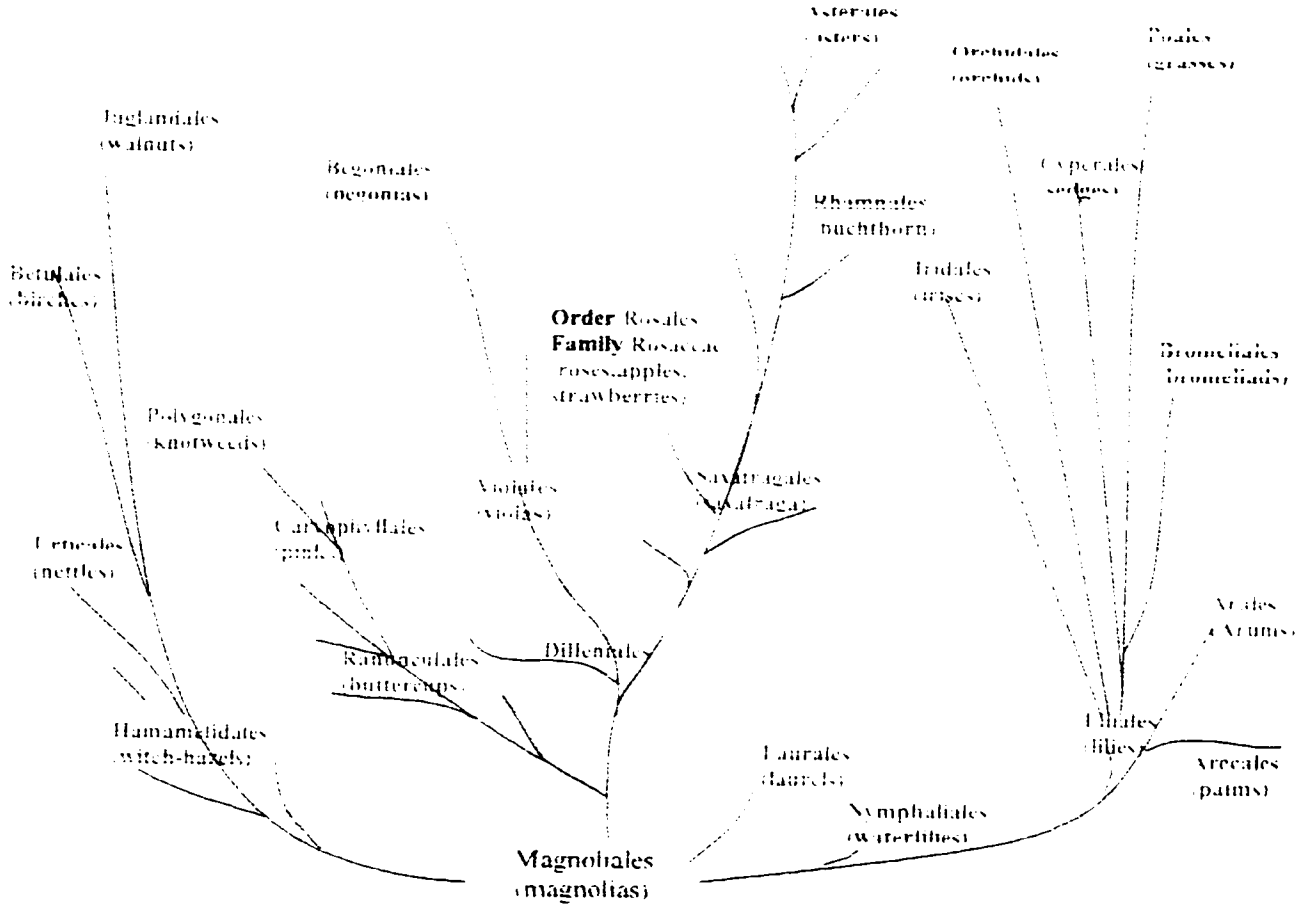
GROUP 2: (chromosome number 17) 1 Subfamily

1. **Maloideae** (*Amelanchier, Aronia, Chaenomeles, Cotoneaster, Crataegus, Cydonia, Eriobotra, Malus, Mespilus, Photinia, Pyracantha, Pyrus, Quillaja, Raphiolepis, Sorbus, Stanvaesia)*

The family's distribution is worldwide but is centered in northern temperate regions. Within Rosaceae are 122 genera and 3,370 species (Heywood 1978). Within the Rosaceae is the order Rosales which contain two main groups. Group 1 contains 4 subfamilies; Spiraeoideae, Neuradoideae, Prunoideae and Rosoideae. Within Rosoideae are 5 tribes; Ulmarieae, Kerrieae, Sanquisorbeae, Roseae and Potentilleae in which *Fragaria* is included. Group 2 contains 1 subfamily, Maloideae.

Perhaps the closest family to the Rosaceae is the Saxifragaceae (Scagel *et al.* 1965, Takhatjan 1966, 1997). Distinguishing characters of the Rosaceae include the presence of stipules, the flowers which are perigynous and seeds with no endosperm. (Benson 1962). The fossil records show the Rosaceae to be among the most ancient of dicotyledons and its "anthecology and general structure" suggests that it is one of the more primitive (Fig 1).

Figure 1. A simplified replication of the dendrogram of the phylogenetic relationship among major orders of flowering plants showing position of order Rosales in one of the three main lines of flowering plant evolution (Takhtajan, 1966, 1980, 1997).


Asterales
(asters)
Orchidales
(orchids)
Poales
(grasses)
Juglandales
(walnuts)
Begoniales
(begonias)
Rhamnales
(buckthorns)
Cyperales
(sedges)
Betulales
(birches)
Iridales
(irises)
Order Rosales
Family Rosaceae
(roses, apples,
strawberries)
Bromeliales
(bromeliads)
Polygonales
(knotweeds)
Violales
(violas)
Saxifragales
(saxifrages)
Caryophyllales
(pinks)
Urticales
(nettles)
Arales
(Arums)
Ranunculales
(buttercups)
Dillenidales
Hamamelidates
(witch-hazels)
Liliales
(lilies)
Laurales
(laurels)
Arecales
(palms)
Nymphaliales
(waterlilies)
Magnoliales
(magnolias)

1.2 Taxonomic History of *Fragaria*:

In 1623, in the Pre-Linnaeus era, 3 strawberry species namely *F. vesca, F. viridis* and *F. moschata* can be traced in lineage (Bauhin 1623). In 1557, *F. chiloensis* was introduced to Cuzco, Peru and later reported by Alonso de Ovalle (1646) and Frezier (1716) to be cultivated in Chile (Popenoe 1921, Darrow 1957). *"Fragaria chiliensis"* was first described by Frezier (1716) based on material he collected at Concepción, Chile (Staudt 1962). By 1738, all strawberries were thought to belong to a single species, *F. flagellis reptans* (Linnaeus, Hortus Cliffortianus, 1738). Later, Linnaeus (1753) was able to recognize 3 species. By 1766, Duschesne's publication titled "Histoire Naturelle des Fraisiers" became a "starting point of modern [*Fragaria*] taxonomy" (Staudt 1962). By 1854, *F. chiloensis* was reported along the coast of Chile and believed to be "indigenous to Chile and the Sandwich Islands (now known as the Hawaiian Islands)" (Gray 1854, Vilmorin 1906). By 1827, it was reported in North America (Chamisso & Schechlendahl 1827). *Fragaria* X *ananassa*, the hybrid of *F. virginiana* and *F. chiloensis* ssp. *chiloensis* is believed to have developed in Europe in the early 1700's (Miller 1759, Wilhelm & Sagen 1974). It is estimated that between 1714 and 1759 was the time period for cultivation or the establishment and dispersal from gardens (Darrow 1966, Staudt 1961).

Within the genus of *Fragaria* are four groups which include the diploids (2n=14, distributed in Eurasia and America), tetraploids (2n=28, east and southeast Asia), hexaploids (2n=49, Europe), and the octoploids (2n=56, North and South America with one disjunction in the Far East (southern Kuriles Islands)) (Staudt 1989).

The only North American monograph on *Fragaria* was that done by Rydberg (1908) in which he recognized 27 species, and no separate subspecies within *F. chiloensis*. As currently

understood, the North American species include the octoploid *F. virginiana* Duch., the meadow strawberry, which occurs from Louisiana and Georgia to Hudson Bay and the Dakotas to Quebec and Newfoundland. Octoploids presumably came from ancient diploids (Darrow, 1966). The only other species in eastern North America is the more slender diploid, *F. vesca*, found from Newfoundland and Manitoba to Virginia (Darrow, 1966). *Fragaria chiloensis*, also an octoploid, is confined to the coastal beaches from Santa Barbara, California north to the Aleutian Islands. The existence of a putative hybrid in British Columbia has been suggested by Staudt (1989), ie. *F.ananassa* nm. *cuneifolia* (Nutt. Ex Howell) with distribution believed extending from Vancouver Island along the Pacific coast south to Fort Bragg, California (Staudt 1989).

At present, 18 species of *Fragaria*, and 4 subspecies of *F.chiloensis* are recognized (Staudt 1962, 1989) including ssp. *pacifica* (Staudt) from the Pacific coast of North America with spreading hairs, ssp. *lucida* (E. Vilm.) from the Pacific coast of North America with ascending hairs, ssp. *chiloensis* Duchesne from South America and ssp. *sandwicensis* (Decaisne) Staudt from the Hawaiian Islands.

In addition to the work of Staudt (1962, 1989), a small number of recent taxonomic studies have focussed on specific problems in North American *Fragaria*. For example, Bringhurst & Khan (1962) established the occurence of hybrids involving *F. chiloensis* and *F. vesca* on the Pacific coast. In addition, Catling (1993), Catling & Cayouette (1994) and Catling *et al.* (1995) clarified the taxonomic status of *Fragaria multicipita* indicating its dwarfed morphology to be a consequence of mycoplasma infection. Also, recent studies of sexual dimorphism in *F. virginiana* from Winsconsin have contributed information useful in classification but were not significantly directed to classification problems (Stahler *et al.* 1995, Ahmadi & Bringhurst 1991).

Recent genetic work on strawberries has mainly focused on the cultivars *F.* X *ananassa* for the Californian market (Hancock *et al.* 1994) or simply on the improvement of techniques to enhance detection and interpretation of codominant RAPD markers (Davis *et al.* 1995). Work on Californian *F. chiloensis* included the identification of strawberry genotypes and evaluation of their genetic relationship using randomly amplified polymorphic DNA (RAPD) analysis (Levi *et al.* 1994) and the examination of population structures in *F. chiloensis* using allozyme markers to measure genotypic differences among fragments in Californian coastal populations (Alpert *et al.* 1993). However, little taxonomic or genetic work is or has been done to examine the pattern of variation within Canadian strawberries and between other subspecies groups.

1.3). Review of species, subspecies, variety and forma concept:

Taxonomic nomenclature continues to possess many inconsistencies for both wild and cultivated plants and there is a need for the increased clarification in the use of infraspecific categories in addition to a need for philosophical discussions of intraspecific taxonomy (Styles 1986, Stace 1986, Hamilton & Reichard 1992). Currently, infraspecific classification is widely used but simply inconsistent in its usage (Yatskievych & Moran 1989, Hamilton & Reichard 1992).

Linnaeus, who founded the binomial system of naming plants, defined "species" in Hortus as "a kind of plant or animal that is distinct from other kinds in marked or essential features, that has good characters of identification, and that may be assumed to represent in nature a continuing succession of individuals from generation to generation" (Linnaeus 1738, Bailey 1933). Since this time, the use of species models have become popular and quite controversial (Andersson 1990, 1992, Nooteboom 1992, Bremer & Eriksson 1992, Grant 1992). Theories include biological,

unitary, ecological, evolutionary, phenetic, cohesion, phytogenetic, autopomorphic, recognition and taxonomic concepts (Riesberg & Brouillet 1994).

Among the important concepts for plants which advanced a few decades ago is the Biological Species Concept which considers species to be "groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr 1969). The Unitary Species Concept considers species as the most "extensive units" where reproductive competition occurs among their parts" (Ghiselin 1974). Ecological Species Concept models species as a lineage which occupies a unique zone (Van Valen 1976). Evolutionary Species Concept considers species to have a single lineage (Wiley 1978). Phenetic Species Concept defines species to be "dense regions within a hyperdimensional environmental space" (Sokal & Crovello 1970).

More recent concepts as shown by recent publications in the last 10 years includes the Cohesion Species Concept which implies the potential for genetic and/or demographic "exchangeability" to model species (Templeton 1989). Phylogenetic Species Concept refers to distinct clusters of organisms which have a "parental pattern of ancestry" (Cracroft 1989). Autopomorphic Species Concept refers to the smallest important lineage deemed worthy of formal recognition (Mishler & Budd 1990). Recognition Species Concept implies the "most inclusive population of individual biparental organisms which share a common fertilization system" (Paterson 1985). Taxonomic Species Concept considers species to be the smallest groups that are consistently distinct and distinguishable by ordinary means (Cronquist 1988). These concepts being somewhat diverse have resulted in an improved understanding yet there have been few major improvements in the past several years in the concept of a species being a group of potentially interbreeding populations with distinctive ecological relationships.

Many authors use the rank "subspecies" for a taxa with greater geographic distinctness instead of "varieties" such as in the cases of African Rubiaceae and the Amazon lilies including *Eucharis* and *Caliphruria* (Verscourt 1987, Chisumpa & Bummitt 1987, Meerow 1989). Some authors classify on the basis of morphological distinctness (Carter 1987, Leach & Williamson 1990). "Subspecies" is often used for taxon with some evolutionary and/or ecological integrity, while "variety" is usually not ecologically distinct but morphological and geographic differences are common (Long 1988, Weitz 1989). However, some impose the opposite approach of ecological distinctness (Hyland 1989). Discrepancies in species and variety usage are found between the US, Canada and Europe, "variety" being the more frequent rank of choice in the US and Canada (Hamilton & Reichard 1992, Catling & Cayouette 1994) and "subspecies" prevailing elsewhere. In North America, "subspecies" is often characterized by morphological distinctness and by the allopatric distribution of these members from the rest of the species whereas "varieties" express only morphological distinctness (Semple 1974). Using this definition, both subspecies and variety rank can be used together as in taxonomic clarification of *Heterotheca sessiliflora* ssp. *echioides* var. *bolanderioides* Semple within the Compositae (Semple 1992). There appears to be some consistency in North America with regards to this usage (Crins *et al.* 1977, Semple & Brouillet 1980, Semple *et al.* 1988). Other authors in North America have used the ranking "variety" to be characteristic of both morphological and geographical distinctness. Problems arise when variation of a variety is not consistent throughout the range as in the case of the Grass Pink Orchid, *Calopogon tuberosus* var. *latifolius* (Catling & Lucas 1987).. The concept of variety in this case has been described as being "useful only if one or few morphological differences are discontinuous that have a geographical basis" This disagrees with the "Fernaldian" application of varietal rank which

recognizes varieties as geographically intergrading morphotypes (Fernald 1950). Other authors like Warner and Chinnappa (1986) agree with Cronquist's approach (1981), who stated that taxonomic treatment can be at times "purely a matter of taste."

"Forma" is usually utilized in the traditional sense to refer to "distinct phenotypes of no persistent populational significance" (Cronquist 1988). Namely, formae do not have any geographic and/or phylogenetic significance like subspecies or varieties (Rieseberg & Brouillet 1994), but are often genetically based. Some recognize formae to comprise distinct populations (Carnevali & Ramirez 1990, Killeen 1990). Often, rank transferral of form to subspecies or vice-versa can occur. In some cases, forms have been transferred to higher ranks and the transfer of varieties and subspecies to the rank of form is often useful when the former ranks are inappropriate yet, the taxon still needs a name to facilitate identification and communication.

With a diversity of techniques at its disposal, including recent molecular and statistical advances, biosystematics can resolve problems of relationship to a greater degree and concepts and ranks may be expected in effect, to become increasingly consistent.

CHAPTER 2: Refinement of hypotheses based on morphological characters:

A morphometric comparison of the subspecies of *Fragaria chiloensis*.

2.1.) Introduction:

Four subspecies of *F.chiloensis* are recognized including ssp. *pacifica*, from Pacific coast of North America with spreading hairs, ssp. *lucida* from Pacific coast of North America with ascending hairs, ssp. *chiloensis* from South America and ssp. *sandwicensis* from the Hawaiian Islands. Many inconsistancies can be found within their taxonomy. Some authors recognize the North American subspecies as distinct (Staudt 1962, Douglas et al. 1991) while others do not (Hitchcock & Cronquist 1973, Ertter 1993). When no distinction is given, they are considered simply *"F. chiloensis"* which groups them with the South American species. Additional taxonomic problems arise within the plants from the Hawaiian Islands which have been regarded on occasion identical to South American plants (Hitchcock & Cronquist 1973; Brako *et al.* 1995), as distinct species (Vilmorin 1906) and even as a variety, var. *sandwicensis* Deg. & Deg. 1961 (St. John 1973). The inconsistencies need to be addressed and a better understanding of the intraspecific variation of *F. chiloensis*, especially within subspecies, is required. If subspecies were identical as some authors suggest, there would be no need to protect or utilize plants from different regions, but if they were genetically distinct in different geographical areas, then protection and utilization in crop improvement could be very important. The objective is to complete a morphometric comparison of the four subspecies (*lucida, pacifica, chiloensis, sandwicensis*) so as to develop a refined hypothesis of relationships.

2.2.) Method:

Ninety-five *F. chiloensis* plants were borrowed from the following herbaria: Agriculture and Agri-Food Canada (AAFC), University of British Columbia, Vancouver (UBC), Museum of Natural History, University of Oregon, Eugene (OSC), New York Botanical Gardens, Bronx (NY), Washington State University, Washington (WS), United States National Museum, Washington (US), Herbarium of the University of California at Berkeley and Jepson Herbarium (UC/Jeps), Museo Argentino de Ciencias Naturales, Buenos Aires (BA), Herbario del Instituto de Botanica Agricola, Buenos Aires (BAB), Instituto de Botanica Darwinion, Argentina (SI), Herbario Nacional de Bolivia, Herbario del Museo Nacional de Historia Natural, Chile (SGO) and Herbarium of Bernice P. Bishop Museum, Hawaii (BP). Specimens utilized are listed in Appendix B. Thirty plants were measured from North America representing, ssp. *pacifica* (Fig. 2); 20 plants from North America representing, ssp. *lucida* (Fig. 3); 27 plants from South America representing, ssp. *chiloensis* (Fig. 4) and 18 plants from Hawaii representing, ssp. *sandwicensis* (Fig. 5). The South American plants probably included both wild races and races escaped from cultivation, and those from Ecuador and Peru (Fig. 4) are presumably naturalized following introduction from further south on the coast by Spanish explorers between 1550 and 1700.

Fourteen characters were measured for each subspecies (Table 1). Since we were concerned with evaluating differences between subspecies rather than differences between sexes, characters known to be significantly different between sexes (including perianth size characters) were not included in the analyses (Hancock *et al.* 1980). The tooth distribution ratio was determined as the maximum length of the lamina divided by the distance from the base of the leaflet along the midvein to a point opposite the tip of the lowest tooth. Measurements of the number of hairs and length of

the longest hair on the leaf and the petiole in one mm^2 were made using a camera lucida and a digitizing tablet. Only hairs that originated within the one mm^2 were counted or measured.

F-ratios from analysis of variance (ANOVA) were used to determine and apply the best discriminators for the four groups of taxa in various combinations. A linear discriminant analyses using standardized data (SAS ver 6.0, SAS Inst.Inc., Cary, NY; Statgraphics Plus ver. 7.0, Manugistics 1993, Cambridge MA.) was used to evaluate the relationship between different subspecies. The misclassification rate was calculated using the crossvalidation option in SAS. Crossvalidation treats n-1 of n training observations as a training set. The discriminant functions are determined based on these n-1 observations and then applies them to classify the one observation omitted. In addition, all characters were tabulated with ranges, means, standard deviations, and F-statistics. Plots of the studentized residuals and estimated values were used to determine the extent of the dependence on the mean for some characters. Data which did not meet the assumptions of the ANOVA were transformed using log transformation. Data for petal number and tooth distribution were not transformable and it was necessary to use the non-parametric Kruskal-Wallis test. Most characters were normally distributed and homoscedastic as indicated by Bartlett's Test with the exception of length of hair on lamina, length of petiole, petal number, number of hairs on petiole and tooth distribution. Discriminant analysis assumes multivariate normality and homoscedasticity, but minor violations were not considered serious due to the robust nature of the analysis (Sneath & Sokal 1973). The discriminant analysis was repeated using log transformations to confirm that any heteroscedasticity did not affect the overall results.

Figure 2. Map of distribution *F.chiloensis* ssp. *pacifica* obtained from herbarium specimens included in the study.

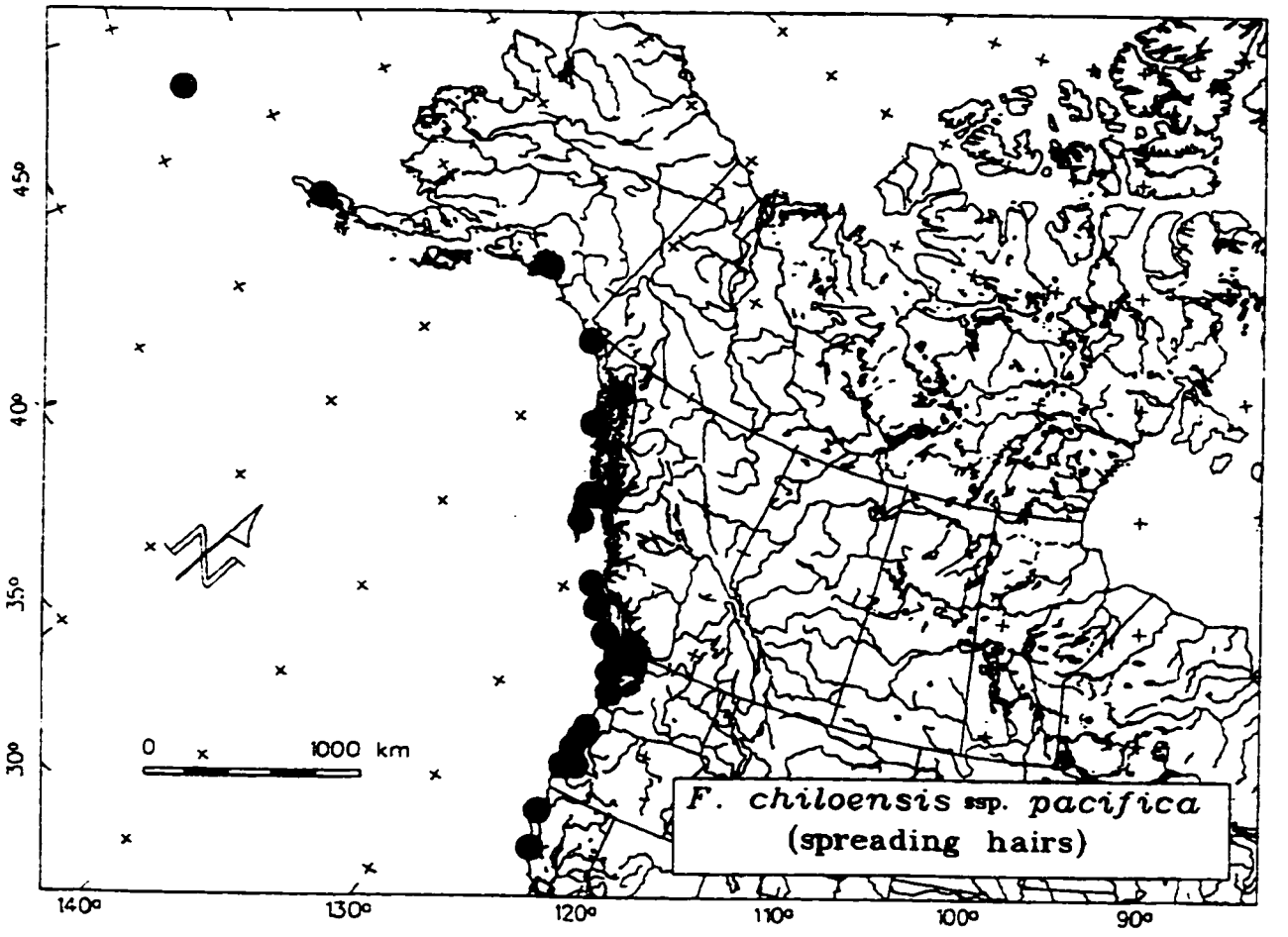

45°
40°
35°
30°
140°
130°
120°
110°
100°
90°
0
1000 km
F. chiloensis ssp. pacifica
(spreading hairs)

Figure 3. Map of distribution of *F. chiloensis* ssp. *lucida* obtained from herbarium specimens included in the study.

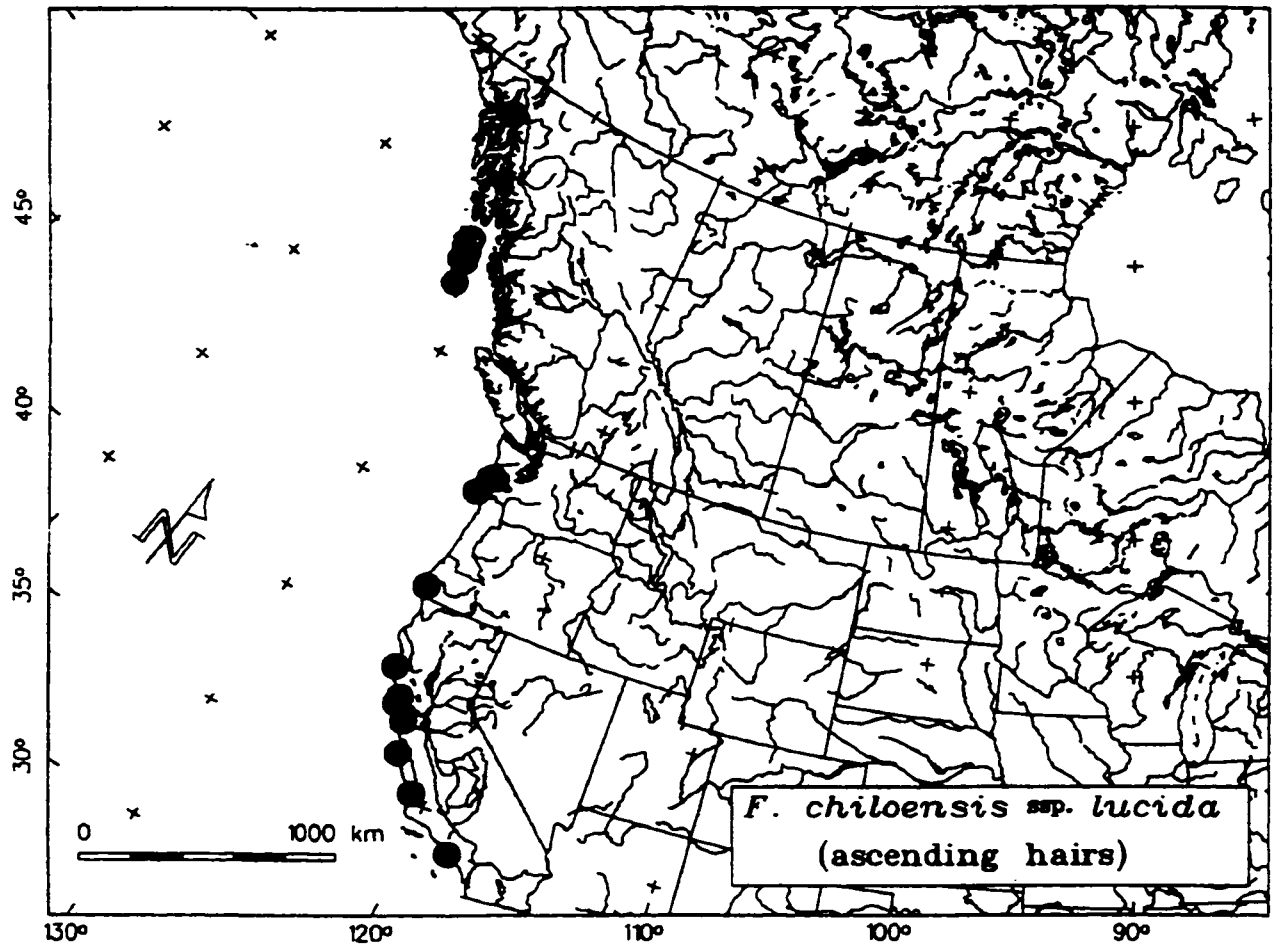

45°
40°
35°
30°
130°
120°
110°
100°
90°
0
1000 km
F. chiloensis ssp. lucida
(ascending hairs)

Figure 4. Map of distribution of South American *F. chiloensis* ssp. *chiloensis* obtained from herbarium specimens included in this study.

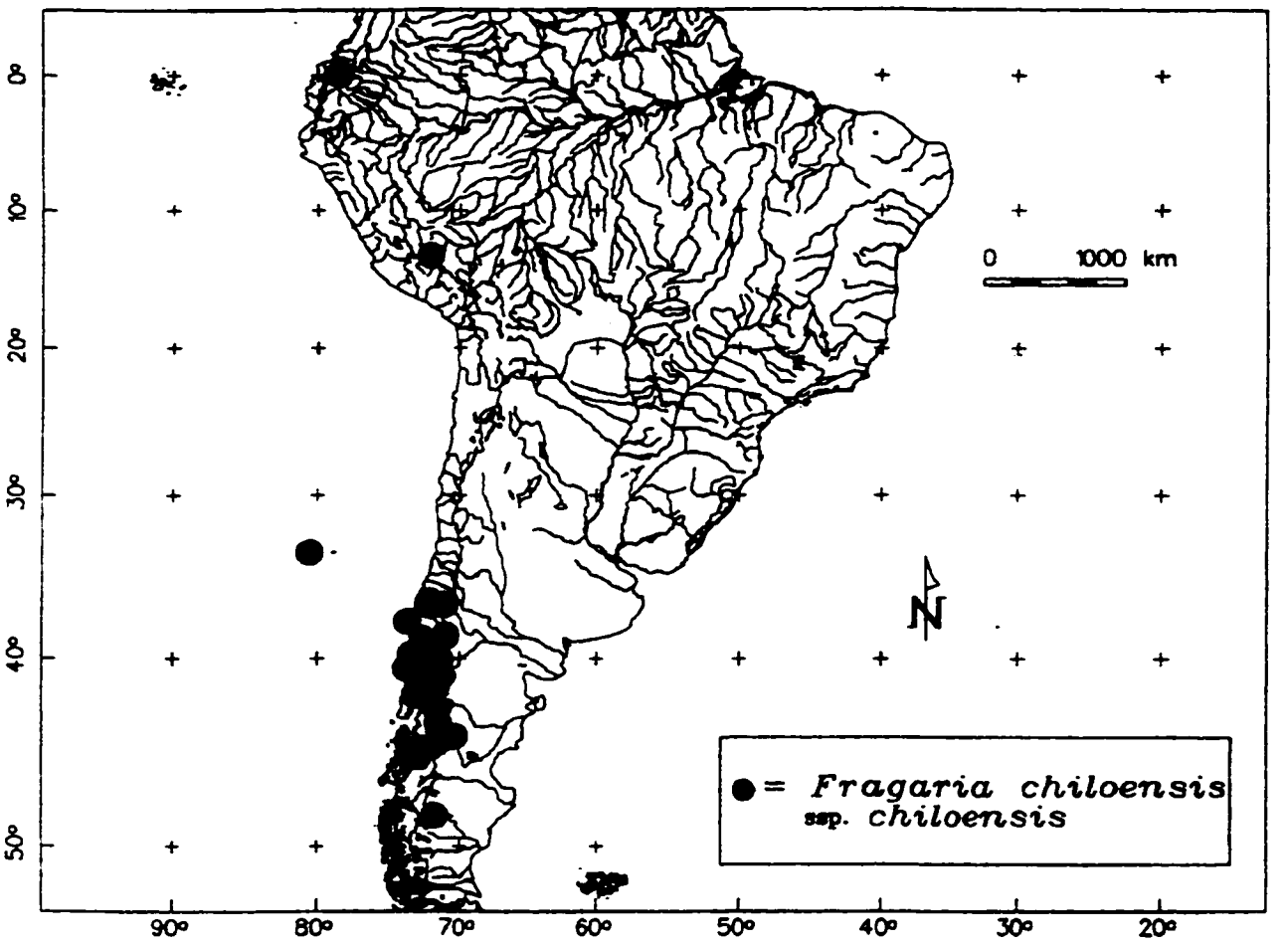
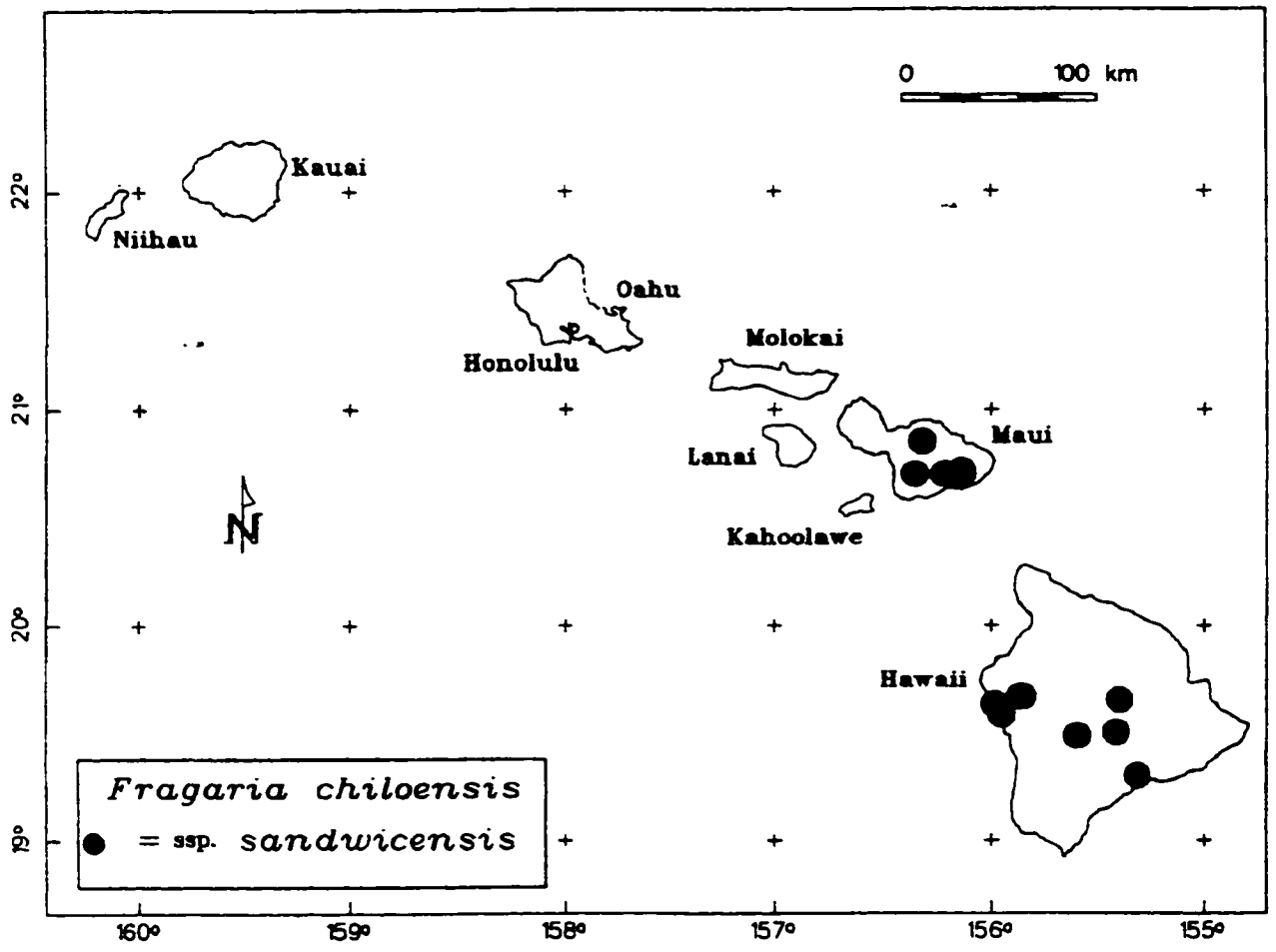

0
10°
20°
30°
40°
50°
90°
80°
70°
60°
50°
40°
30°
20°
0 1000 km
N
= Fragaria chiloensis
ssp. chiloensis

Figure 5. Map of distribution of *F. chiloensis* ssp. *sandwicensis* from the Hawaiian Islands obtained from herbarium specimens included in the study.


0 100 km
Kauai
Niihau
Oahu
Honolulu
Molokai
Lanai
Maui
Kahoolawe
Hawaii
N
Fragaria chiloensis
= ssp. sandwicensis
22°
21°
20°
19°
160°
159°
158°
157°
156°
155°

2.3.) Results:

Plots of the studentized residuals and estimated values revealed only slight dependence on the mean for some characters. The first, second and third discriminant axes accounted for 60.45%, 31.38% and 8.17% of the variation respectively (Fig. 6, $X^2 = 99.27$, $p<0.0001$). With log transformations for the necessary data the axes accounted for 57.36%, 33.67% and 8.97% of the sample variation respectively indicating that heteroscedasticity did not substantially affect the overall results and significance level ($P< 0.0001$). Hawaiian plants were a discrete group separated on the first axis while the second axis only partially separted the South American and North American plants (Fig. 6), and the two North American ssp. overlapped extensively. The ssp. *sandwicensis* appears to have the largest leaves (Table 2). Leaf hairs on the leaflet are longer and more numerous and veins are more numerous. Petiole length of the middle leaflet is also almost always longer in ssp. *sandwicensis* (Tables 1,2).

Misclassification percentages calculated for the four discriminant subspecies groups revealed high error counts for North American and South American groups while Hawaiian group displayed much lower misclassifications; ssp. *lucida* = 75%, ssp. *pacifica* = 50%, ssp. *chiloensis* = 51%, ssp. *sandwicensis* = 11%. The analysis was repeated joining North American groups and resulted in lower overall group misclassifications; North American = 42%, South American = 41% and Hawaiian = 5% (Appendix A).

The ssp. *chiloensis* was largely distinctive by its large number of petals ranging from 6-10 averaging 6-7 in comparison to the other subspecies which have 5-6, averaging 5. The South American and North American plants differ in qualitative characters not used in the analysis such as flower characteristics. Furthermore, a separate discriminant analysis of the North and South

American plants had a chi-square significance value of x^2=64.04 (p=0.00001), supporting recognition of two taxa (Fig. 7).

The North American subspecies were not well separated by any character other than the hair orientation character used to define them. The best additional character for separating these two groups was the number of leaf veins, ssp. *lucida* tending to have more veins, but the overlap was more than 80%. A separate discriminant analysis for these groups had a chi-square of X^2=27.82 (p=0.0150). Although significant, the overlap was extensive and the significance level was much less than in the preceding cases.

Using the characters with the higher F-ratios for separating different combinations of groups (Table 2), the subspecies can be separated using the following key. The ranges given in the key refer to 90% of the sample variation with the bracketted numbers representing extremes.

1a. Leaflet hairs 1.6-3.1 mm long; leaflet length 31-61 mm; veins 6-7, flowers functionally bisexual ..... ssp. *sandwicensis*

1b. Leaflet hairs 0.94-1.6 mm long; leaflet length 13-36 mm; veins 3-6, flowers functionally unisexual or bisexual 2

2a. Plants whitish-green; petals 6-10 3

2b. Plants dark green; petals 5-6 4

3a. Plants large, leaflets mostly longer than 40 mm and wider than 25 mm .....
..... ssp. *chiloensis* f. *chiloensis*

3b. Plants small, leaflets mostly less than 40 mm long and less than 25 mm wide ...
..... ssp *chiloensis* f. *patagonica*

4a. Hairs on leaf stalk ascending ..... ssp. *lucida*

4b. Hairs on leaf stalk spreading ..... ssp. *pacifica*

Figure 6. Discriminant analysis of 4 subspecies of *F. chiloensis*.

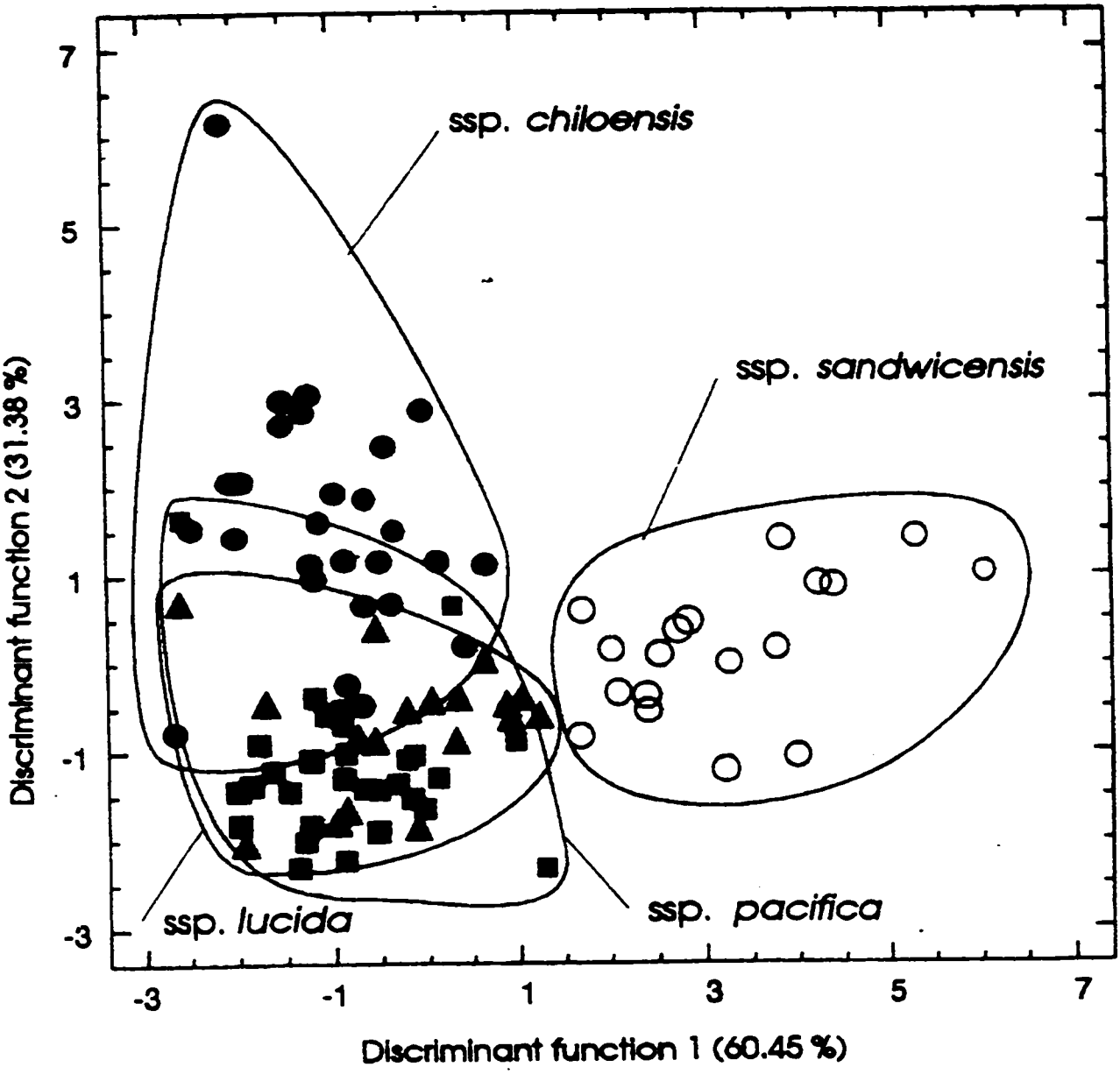

ssp. chiloensis
ssp. sandwicensis
ssp. lucida
ssp. pacifica
Discriminant function 2 (31.38 %)
Discriminant function 1 (60.45 %)
7
5
3
1
-1
-3
-3
-1
1
3
5
7

Figure 7. Discriminant analysis of North and South American subspecies of *F. chiloensis*.

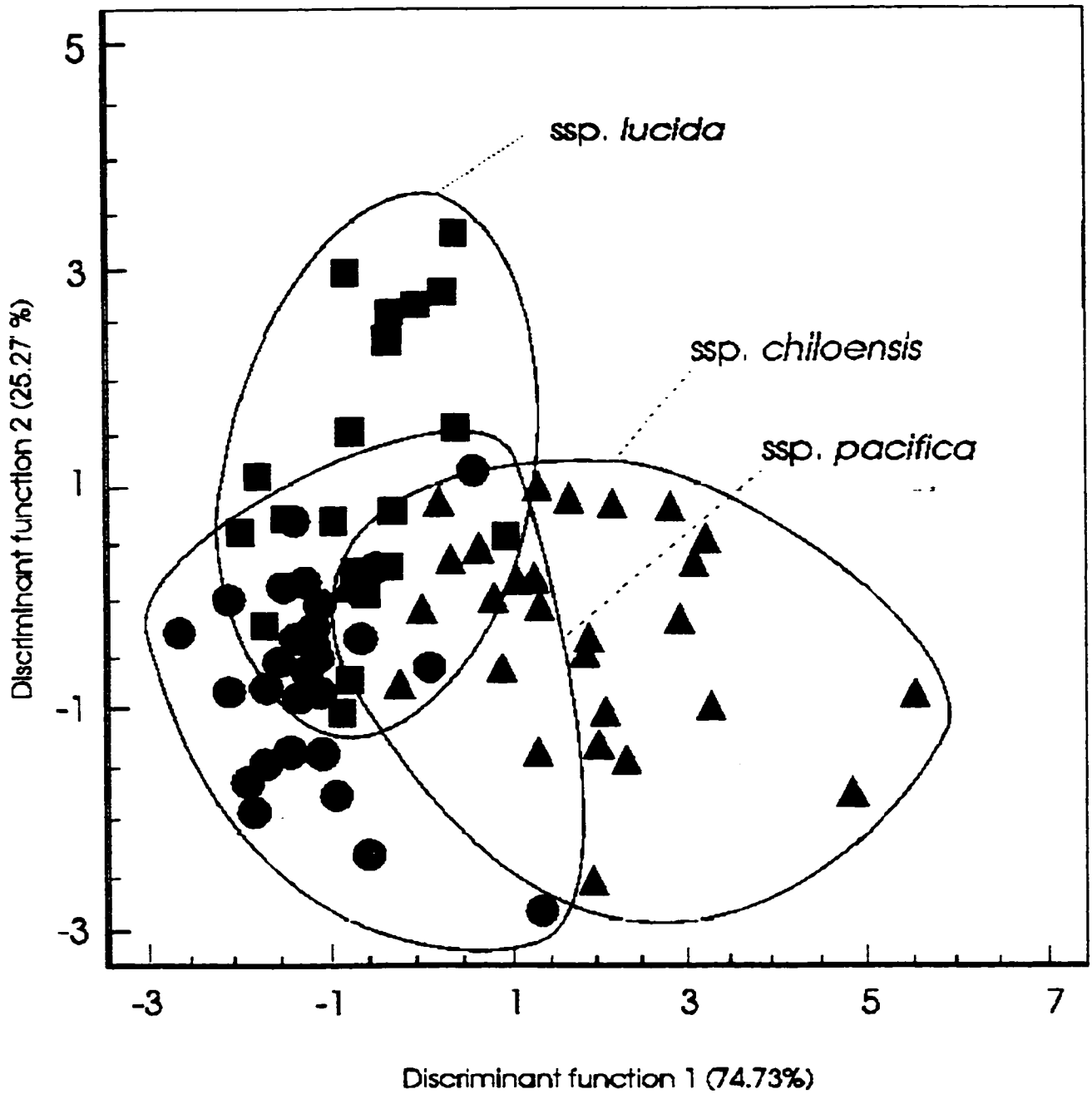

ssp. lucida
ssp. chiloensis
ssp. pacifica
Discriminant function 2 (25.27 %)
Discriminant function 1 (74.73%)
5
3
1
-1
-3
-3
-1
1
3
5
7

Table 1. Characters examined with their ranges, arithmetic means and standard deviations obtained for each subspecies where P= ssp. *pacifica*, L= ssp. *lucida*, C= ssp. *chiloensis* and S= ssp. *sandwicensis*.

CHARACTER	ssp.	Range	Mean	S.D.
1. Total length of lamina (mm)	P	13.00-44.00	27.41	7.84
	L	17.00-42.00	27.48	8.04
	C	16.00-36.00	24.94	6.30
	S	28.00-61.00	41.03	8.60
2. Maximum width of lamina (mm)	P	11.00-39.00	22.62	6.43
	L	11.00-39.00	21.83	6.92
	C	12.00-30.00	19.13	4.93
	S	19.50-43.00	29.22	6.11
3. Width 1/4 distance from base (mm)	P	7.00-25.00	13.58	4.02
	L	6.00-23.00	12.38	4.52
	C	6.00-20.00	10.72	3.39
	S	11.00-27.00	17.67	4.38
4. Width 1/2 distance from base (mm)	P	10.50-37.00	21.05	6.06
	S	9.00-35.00	19.48	6.36
	C	8.50-29.00	16.93	4.99
	S	18.00-43.00	27.42	5.96

CHARACTER	ssp.	Range	Mean	S.D.
5. Width 3/4 distance from base (mm).	P	10.00-34.00	9.68	5.71
	L	11.0-35.00	19.43	6.16
	C	10.00-28.50	17.63	4.58
	S	15.00-36.00	24.72	5.37
6. Number of hairs per mm^2 on lamina	P	9.00-30.00	20.73	6.36
	L	11.00-30.00	22.55	5.91
	C	14.00-42.00	27.04	7.59
	S	11.00-39.00	23.44	5.87
7. Length of longest hair on leaflet (mm)	P	0.98-1.89	1.29	0.22
	L	0.99-1.82	1.37	0.27
	C	0.94-1.72	1.41	0.21
	S	1.35-3.15	1.89	0.49
8. Number of veins	P	3.00-6.00	4.37	0.72
	L	4.00-8.00	4.95	1.19
	C	3.00-6.00	4.59	0.80
	S	5.00-7.00	6.33	0.77
9. Number of teeth	P	3.00-6.00	4.20	1.06
	L	3.00-6.00	4.50	0.95
	C	3.00-7.00	4.37	1.07
	S	4.00-7.00	5.50	0.79

CHARACTER	ssp.	Range	Mean	S.D.
10. Length of petiole (mm)	P	0.73-6.52	2.67	1.61
	L	0.62-7.42	3.24	1.76
	C	0.87-6.99	2.78	1.45
	S	1.90-14.00	6.23	3.84
11. Petal number	P	5.00- 7.00	5.13	0.43
	L	5.00- 6.00	5.10	0.31
	C	5.00-10.00	6.48	0.98
	S	5.00- 6.00	5.17	0.38
12. Tooth distribution	P	1.18-2.00	1.54	0.24
	L	1.15-3.45	1.82	0.55
	C	1.08-2.90	1.56	0.44
	S	1.38-2.26	1.79	0.26
13. No.of hairs in one mm^2 on leaf petiole	P	8.00-34.00	21.64	7.37
	L	11.00-30.00	20.10	4.88
	C	11.00-43.00	26.67	7.49
	S	11.00-32.00	19.39	6.25
14. Length of longest hair in grid on leaf petiole (mm)	P	1.05-2.05	1.52	0.22
	L	0.86-1.97	1.42	0.32
	C	0.85-2.06	1.53	0.26
	S	1.50-2.94	1.93	0.38

Table 2. Characters examined with their F-ratios from one-way ANOVA or *Kruskal-Wallis Test Statistic obtained for each subspecies where ssp. *pacifica*, ssp. *lucida*, ssp. *chiloensis* and ssp. *sandwicensis*.

CHARACTERS	F-Ratio 4ssp.	F-Ratio sandwicensis and other groups	F-RATIO North & South American Plants	F-RATIO 2 North American ssp.	Probability for 4 ssp. Groups
1.Total length of lamina (mm)	18.10	52.52	2.02	0.90	<0.0001
2. Maximum width of lamina (mm)	10.12	24.60	4.81	0.17	<0.0001
3. Width 1/4 distance from base (mm)	11.10	24.75	6.33	0.98	<0.0001
4. Width 1/2 distance from base (mm)	12.10	27.56	6.40	0.78	<0.0001
5. Width 3/4 distance from base (mm)	6.31	16.62	2.26	0.02	0.0006
6. Number of hairs per mm^2 on lamina	4.52	<0.001	12.12	1.04	0.0053
7. Length of longest hair on leaflet (mm)	16.62	45.64	2.82	1.01	<0.0001
8. Number of veins	21.30	56.38	0.01	4.68	<0.0001
9. Number of teeth	6.98	19.99	0.04	1.04	0.0003

CHARACTERS	F-Ratio 4sp.	F-Ratio sandwicensis and other groups	F-RATIO North & South American Plants	F-RATIO 2 North American sp.	Probability for 4 sp. Groups
10. Length of petiole (mm)	7.69	22.03	0.04	1.04	0.0001
11. Petal number	*9.84	*3.58	*46.82	*0.002	0.0199
12. Tooth distribution	*12.63	*7.10	*2.65	*2.58	0.0055
13. No. of hairs in one mm^2 on leaf petiole	4.59	3.73	11.93	0.67	0.0049
14. Length of longest-hair in grid on leaf petiole (mm)	11.73	27.65	0.68	2.50	<0.0001

2.4.) Discussion:

The clear separation of the Hawaiian subspecies supports its recognition at the subspecies level while only partial separation of the South and North American plants were obtained from the discriminant analysis. However, South American plants are pale in colour whereas North American plants are deep green. This difference was maintained in plants from South America and North America plants grown side by side under uniform conditions. Furthermore, the South American plants are not restricted to the coastline like the North American plants and ascend to relatively high elevations in the mountains which suggests ecological differences. Despite some overlap in the morphological-based groups, it is reasonable to maintain the South American subspecies on the basis of these differences.

In contrast, the two North American subspecies overlap extensively and there seems little reason to maintain them at the rank of subspecies. Although there is some phytogeographic separation, namely the ssp. *lucida* being the only one occurring in the southern limit in San Luis Obispo county, California and the ssp. *pacifica* being the only one occurring near the northern limit near the Aleutian Islands (Staudt 1989) (Fig 2,3), there is extensive geographical overlap.

In summation, the four subspecies of *F. chiloensis* appear to best be treated taxonomically as three major groups with the North American plants comprised of two minor groups. All of these groups, but in particular the evidently distinctive Hawaiian and North American subspecies, not presently used in the development of most strawberry cultivars, have the potential to contribute to an improved strawberry crop. This result may be regarded as the best hypothesis of relationship that is readily obtained and available and represents a refinement over the otherwise poorly supported classification to four taxa. This hypothesis requires further exploration since it is based

on a relatively small sample and included a relatively small set of characters derived only from dry specimens. Further exploration using a different approach of molecular characterization with different individuals will likely provide the best approach to clarifying any uncertainties.

Chapter 3. - Modification of a CTAB DNA extraction protocol for mature Strawberry leaves containing high polyphenol and polysaccharide components.

3.1.) Introduction:

DNA extraction from berry plants and other plants containing sticky and resinous materials has been difficult in the past (Webb & Knapp 1990; Varadarajan & Prakash 1991; Fang *et al.* 1992; Collins *et al.* 1992). In strawberries, the presence of secondary metabolites such as polyphenols, tannins and polysaccharides inhibit enzyme action. Polysaccharides are visually evident in DNA extracted by their viscous glue-like texture and make the DNA unmanageable in pipetting and unamplifiable in the polymerase chain reaction (PCR) by inhibiting Taq polymerase activity (Fang *et al.* 1992). When young expanding leaf and shoot material is limited or the plant is not undergoing active shoot elongation at time of collection, DNA purity becomes increasingly difficult. DNA obtained from material past the budding stage has been shown to be difficult to extract and unstable for long-term storage (Lodhi *et al.* 1994). With maturity, leaves contain increased quantities of polyphenols, tannins and polysaccharides. Dealing with such components in mature leaves becomes necessary when younger expanding leaves and shoots are not available during the time of collection.

Numerous unsuccessful attempts to PCR amplify *Fragaria* DNA resulted using other documented methods, including, Dellaporta *et al.* (1985), Saghai-Maroof *et al.* (1984), Doyle & Doyle (1987), LaRoche (1992), Oard & Dronavalli (1992), Wang *et al.* (1993), Richards *et al.* (1994) and Davis *et al.* (1995). As a result, it became necessary to devise a protocol for DNA

extraction which would provide a DNA yield suitable for PCR, especially for random amplified polymorphic DNA (RAPDs), from fully developed expanded leaf tissue which would be consistent for many different wild and cultivated species of strawberries of both diploid and octoploid genotypes.

Based on the hypothesis that an efficient extraction method for mature strawberry leaves could be developed using NaCl and PVP to remove excess polysaccharides and polyphenol components, the objective is to obtain successful amplification of DNA in polymerase chain reaction (PCR) that is stable and reproducible.

3.2) Materials and Methods:

Sixty strawberry plants of both wild and cultivated Canadian octoploid species (2n=56) including *F. chiloensis* (L.) Dcne., *F. virginiana* Dcne, *F. virginiana* forma *multicipita* (Fern.) Catling & Cayouette, *F.* X *ananassa* nm. *cuneifolia* (Nutt.) Staudt, and one diploid species (2n=14), *F. vesca* L., were used, approximately 5-12 plants per species. The modification of Doyle & Doyle (1987) CTAB (cetyltrimethylammonium bromide) extraction includes in the buffer high NaCl concentrations, to remove polysaccharides (Lodhi *et al.* 1995), and polyvinyl pyrrolidone (PVP), to remove polyphenols. (Maliyakal's 1992). In order for the DNA product to be RNA free and PCR amplifiable, an extended RNase treatment was required for a one hour duration with an additional phenol chloroform cleaning (Saghai-Maroof *et al.* 1984) to remove any excess proteins. These main steps provide the key to success for DNA removal from mature strawberry leaves.

Examination of polymorphism through use of primers with arbitrary sequences (RAPD) was done successfully with 16 different primers after initial screening of approximately 100 primers.

Primers that produced background effects, namely poor band definition due to visualized smearing of bands were avoided (appendix B). The 25 µL volume PCR reactions contained 1X buffer (100 mM Tris-HCl, 100mM KCl, pH 8.3), 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM primer (set #8 UBC - University of British Columbia), 2u Stoffel fragment (reagents supplied by Perkin Elmer), 1 to 1.5ng DNA, and were placed in a MJ Research Inc. PTC100 Thermocycler. The thermal cycling included one cycle at 15 seconds of denaturation at 94°C, 30 seconds annealing at 35°C and one minute of primer extension at 72°C for 30 cycles. On 30 th cycle, primer extension is increased to 7 minutes. PCR amplicons were then run for 3 hours on a 1.5% 3-4mm at 100 volts for best results. This provided adequate band separation with minimal loss of band intensity.

In order to help standardize the RAPD molecular technique for the characterization of germplasm collections, a summary table has been provided as suggested in a current paper by Lowe *et al.* (1996) (Appendix C).

The Extraction Protocol

Solutions Required:

Chloroform:Octanol 24:1 (v/v)

5M NaCl

TE Buffer: pH 8.4 (10 mM Tris-HCl & 1 mM EDTA)

RNase A (10 mg/mL)

Proteinase K (1mg/mL) made fresh before use

Chloroform - Phenol saturated in TE (1:1)

Polyvinylpyrrolidone (PVP) Sigma P -9003-39-8 (40,000)

Extraction buffer: 100 mM Tris-HCl, 1.4M NaCl, 20mM EDTA pH 8.0, 2% CTAB 0.3% ß-mercaptoethanol (added just before use)

DNA Extraction

· Collect leaves of *Fragaria*, freeze in liquid nitrogen and store at -70°C until use. Grind 0.5 g of leaves using mortar and pestle in the presence of liquid nitrogen until finely ground. Transfer frozen ground leaf tissue to 15 mL polypropylene centrifuge tubes.

· Add 5 mL of 60°C extraction buffer and 50 mg PVP/ 0.5 g leaf tissue. Mix by inversion and incubate in 60°C oven (shaking) for 25-60 minutes.

· Remove from heat and let cool to room temperature for 4-6 minutes.

· Add 6 mL of chloroform:octanol (24:1) and mix by inversion to form an emulsion.

· After mixing thoroughly, spin at 3000 rpm for 20 minutes in a tabletop centrifuge at room temperature.

· Transfer top aqueous solution to new 15 mL centrifuge tubes using wide-bore pipette tip. Repeat chloroform-octanol extraction to remove cloudiness (PVP's) in aqueous phase. [1]

· Add 1/2 volume of 5M NaCl to the final aqueous solution recovered. [2] Mix well. Add two volumes of cold (-20°C) 95% ethanol. Mix by inversion. If required, place in freezer (-20°C) for 10 minutes to accentuate precipitation. The solution may be left at 4-6°C to precipitate overnight.

· Spin at 3000 rpm for 6 minutes. [3]

· Pour off supernatant and wash pellet with cold (0-4°C) 70% ethanol. Dry pellet in 37°C oven or vacuum until dry (~1 hour).

· Dissolve in 300 µL TE overnight at (4-6°C-refrigerate). [4] Transfer to 1.5 mL Eppendorf tubes. [5]

· Add 3µL RNase A (10 mg/mL) and incubate in 37 °C water bath for approximately 1 hour. Add 3µL Proteinase K (1mg/mL) and incubate at 37 °C for 15-30 minutes.

· Add 150 µL of phenol and 150 µL of chloroform to each Eppendorf tube.

· Vortex briefly and spin (in microfuge) at 14000 rpm for 10-15 minutes. Collect upper layer in new 1.5 tube. Add 50 µL TE to phenol phase. Vortex, spin and remove upper layer and add to sample.

· Add 1/10 vol. 2 M NaAcetate and 2 vol. absolute ethanol and mix.

· Leave overnight in freezer (-80°C). Spin at 14000 rpm for 10-20 minutes. Drain and wash with 70% ETOH. Remove ETOH.

· Vacuum dry tubes. Add 100-200 µL TE. Allow time for complete resuspension (recommended overnight at 4°C).

·DNA concentrations were measured using a Hoefer Quanta 200 Fluorometer and 0.5 ng DNA dilutions were prepared for PCR use.

Notes

1. Be careful to remove only top aqueous solution and not white band at interface. (Repeat this step until no longer cloudy due to the presence of PVP bound polysaccharides . Twice is usually sufficient).

2. In some plants a second salt precipitation may be useful.

3. DNA pellets should now be visible in all tubes.

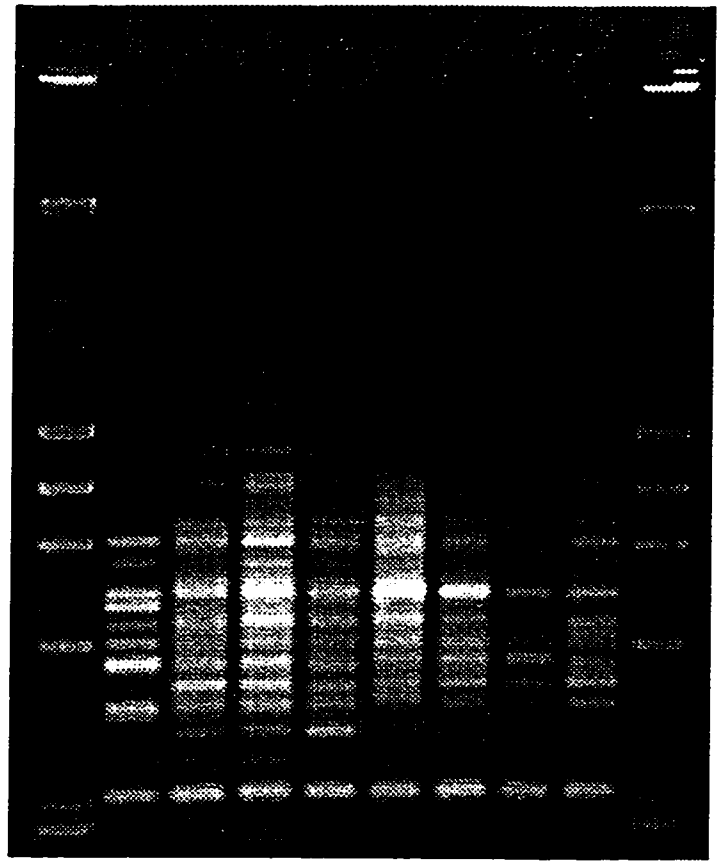
4. When dissolved, some DNA may still be slightly viscous but easily pipetted.

5. Products at this point in the protocol are usually not PCR amplifiable.

3.3) Results:

DNA concentrations were measured for the sixty samples and the average yields and ranges (µg/g leaf tissue) are *F. vesca* (20, 10-30); f.*multicipita* (30, 15-45); *F.virginiana* (Gaspé) (20, 10-25), (Petawawa, 84, 20-120); *F.chiloensis* (V.I., 71, 35-90), (B.C., 52, 10-90); *F. ananassa* (wild, 32, 30-45), (cultivated, 28, 15-45). PCR amplification was successful using this procedure using quantities as small as 0.5ng in a 25 µl reaction (Fig. 8). This supports the hypothesis that with the addition of NaCl and PVP to remove excess polysaccharides and polyphenol components, successful DNA extraction is obtained which results in stable, PCR amplifiable DNA. When DNA amounts were increased beyond 5 ng in the PCR reaction, DNA bands became less distinct and were less reproducible likely do to increased quantities of inhibiting components. Although secondary metabolites are likely not completely removed, the DNA template was pure enough to allow PCR amplification. In addition, such DNA yields are large enough to permit as many as 1.4×10^5 PCR reactions or a number of RFLP applications.

Figure 8. PCR amplicons on a 1.5% agarose gel with primer UBC8-751 (left to right). (1) Marker lambda HindIII + phiX174 digested with Hae III, (2) *F. vesca* L., (3) *F. virginiana* f. *multicipita* (Fern.) Catling & Cayouette - from Gaspé, (4) *F. virginiana* Dcne. from Gaspé, (5) *F. virginiana* from Petawawa, Ontario (6) *F. chiloensis* (L.) Dcne. from S. Vancouver Island, (7) mid coast B.C., (8) *F. ananassa* nm. *cuniefolia* (Nutt.) Staudt - from V.I wild, (9) cultivated, (10) Marker.



3.4). Discussion:

This DNA extraction protocol is relatively fast since a lengthy ultracentrifugation is not required as in some other PVP protocols (Maliyakal 1992). PVP, a high molecular weight water soluble chemically inert solid polymer, was shown to remove the polyphenols while maintaining a higher yield than polyvinyl polypyrrolidone (PVPP), an insoluble cross linking polymer. The PVP forms a complex with polyphenols through hydrogen bonding allowing them to be separated from the DNA, reducing levels of polyphenol in the product (Maliyakal 1992). Although PVPP is useful in improving stability of enzymes by removing phenolic impurities (Sigma Chemical Co. 1993), it yields significantly less than PVP from fully expanded leaves.

The technique of Lodhi *et al.* (1994) utilizing PVPP gave very low DNA yields, 2-3 μg/g leaf tissue, with fully expanded leaves, and PCR amplification was not consistently successful for all strawberry species. Even when young expanding leaves and shoot material were examined using this protocol, yields reported by Lodhi *et al.*(1994) for other berry plants (546-1130 μg/g) were not reached.

The method of Jobes *et al.* (1995) utilizing PVP, NaCl, LiCl and SDS (sodium dodecyl sulfate) is time consuming. We were able to obtain equivalent results with the exclusion of the SDS cleaning and LiCl which is used for RNA removal to prevent residual ribonucleosides acting as primers in the thermal reaction (Storts 1993). We found an extended RNase treatment of 1 hour was sufficient to degrade the RNA into small ribonucleosides which were not detectable by gel electrophoresis. Remaining ribonucleosides would be in very small amounts and would have to compete with large quantities of added primers in the RAPD reaction making ribonucleoside primer interference highly unlikely.

Other protocols proved unsuccessful in removing secondary inhibiting components in all species including a NaOH technique (Wang *et al.* 1993); a protocol developed for maize (Dellaporta *et al.* 1984, Oard & Dronavalli 1992); a CsCl purification (Richards *et al.* 1994); the Elu-Quik (Scheicher & Schuell) method (LaRoche 1992) and various CTAB extractions (Doyle & Doyle 1987, Saghai-Maroof *et al.* 1984, Davis *et al.* 1995)).

Unlike all other protocols mentioned above, our relatively fast, inexpensive, consistent protocol produced very acceptable DNA yields from extracted mature leaf tissue for all *Fragaria* species assayed. All DNA was stable and successfully PCR amplifiable requiring only 0.5 ng DNA quantities in 25 μL reactions, both before and after 21 months of storage at -20 °C. In addition, RAPD profiles were shown to be reproducible for at least 5 plants examined from each location for each species. This technique has potential to be an effective protocol for DNA extraction using mature leaf tissue for strawberries and perhaps other species in the family Rosaceae with high polysaccharides and polyphenols secondary components when sufficient young leaf tissue is not available.

CHAPTER 4: The assignment of taxonomic ranks based on the examination of the relationships of the two North American *Fragaria chiloensis* ssp. *pacifica* and ssp. *lucida* to the South American ssp. *chiloensis*.

4.1) Introduction:

Morphometric analysis (Chapter 2) suggested that ssp. *chiloensis* from South America was different from the two North American subspecies, although there was some overlap in the limited set of morphological characters used in the analysis. Based on the first hypothesis that the North American *F. chiloensis* ssp. *pacifica* is best recognized at the level of forma, and that the North American plants of *F. chiloensis* are distinct from the South American plants and warrant subspecies rank (Chapter 2), the objective is to compare patterns of variation in random amplified polymorphic DNA of North American and South American taxa so as to determine whether variation is greater within or between these groupings.

4.2.) Methods:

The analysis included 35 plants of which 20 were from North America. These were provided by the Canadian Clonal Genebank (Harrow) and represented collections from throughout coastal British Columbia made in 1984 and 1985 by staff of the University of British Columbia (Luffman & MacDonald 1992). These collections represent approximately one half of the range of each North American subspecies (Table 3). Fifteen of the North American plants were referable to ssp. *pacifica* with spreading hairs on leaf stalks, and five were referable to ssp. *lucida* with

ascending hairs. The fifteen plants from South America, referable to ssp. *chiloensis*, were provided by the U.S. National Plant Germplasm Repository in Corvallis, Oregon, and were collected by Cameron *et al.* during expeditions to Chile in 1990 and 1992 (Cameron *et al.* 1991, 1993).

Plants were grown in eight inch pots in the greenhouse at the Central Experimental Farm, Ottawa. DNA extraction from fresh leaf tissue and the polymerase chain reaction (PCR) was carried out as described in Chapter 3. One hundred primers from UBC set #8 were screened. Twelve primers were selected which revealed adequate polymorphism within and between the three subspecies.

Replicates of two to three for all plants were repeated for each primer to check consistency. Band fragments that possessed within or between group variation were scored as present (1) or absent (0). If band was visible and 100% reproducible for all replicates it was scored as (1). Effects of co-migration and misalignment were minimized by the use of a ruler used to align the marker in first lane to the marker in last lane. Data was analysed using UPGMA clustering of Jaccard's coefficients (NTSYS ver. 1.70, Rohlf 1992) (Jaccard 1901, 1908, Sokal & Rohlf 1995). A cophenetic correlation coefficient was calculated through comparison of the Jaccard's matrix with the cophenetic matrix obtained from the phenogram.

4.3) Results:

From the 100 primers screened, twelve primers produced banding patterns that were informative to detect polymorphism within the different site locations: UBC743 (5'CCACCCACAC), UBC746 (5'GGGTGTTGGG), UBC748 (5'CCCTTCTCCC), UBC756 (5'CCCTCCTCCT), UBC759 (5'CCAACCCACC), UBC772 (5'CCCACCACCC), UBC785

(5'CACCCAACCA), UBC790 (5'GGGTGTGGTT), UBC792 (5'CAACCCACAC), UBC795 (5'TGGTGTGGGT), UBC796 (5'AGAGGGAGGA) and UBC798 (5'GAGAGGAAGG). The size of amplification products varied from 230 to 1500 bp in the 62 fragments scored. Most polymorphism was present in fragments ranging from 400 to 800 bp. The number of band fragments for each primer varied. Variation in the North American and South American plants can be seen using primer UBC8-792 (Fig.9). In this case, a 680 bp is unique in South American plants while a 370 bp fragment is unique to the North American plants. Within group variation is shown by the presenee or absence of a 400 bp fragment.

From the total of 62 polymorphic bands (see appendix), twenty bands from nine primers were unique to the North American subspecies; UBC743 (840, 720 bp), UBC748 (780, 740, 720 bp), UBC756 (1150, 840 bp), UBC759 (1500, 700, 690, 620 bp), UBC772 (540 bp), UBC790 (560, 500, 350 bp), UBC792 (370 bp), UBC796 (450 bp) with only two being unique to ssp. *lucida*, UBC759 (720, 550 bp). Thirteen bands from six primers were unique to the South American; UBC743 (870, 700, 290,260 bp), UBC746 (720 bp), UBC759 (1450, 680, 640 bp), UBC790 (520, 480 bp), UBC792 (740, 680 bp), UBC795 (680 bp). All other band fragments scored revealed within group variation. All replicates produced consistent banding fragments.

The phenogram (cophenetic correlation, r=0.99) based on UPGMA clustering of Jaccard's coefficients revealed a clear division between North American and South American plants (Fig. 10) (sim. index = 0.16) but a much more limited discrimination was observed between the two North American subspecies (0.64-0.88). One plant referable to ssp. *lucida* (L2, see appendix) was more closely related to ssp. *pacifica* (P10) than all other ssp. *lucida* plants. Less within group variability is suggested in the South American ssp. *chiloensis* than in the North American subspecies (Fig 9).

Figure 9. PCR amplimers on a 1.5% agarose gel with primer UBC8-792 (left to right): λ+ϕ marker (λ digested with Hind III+ϕX174 digested with Hae III), ssp. *pacifica* (P1, P2), ssp. *lucida* (L1, L3), ssp. *chiloensis* (C1, C2), λ+ϕ marker.

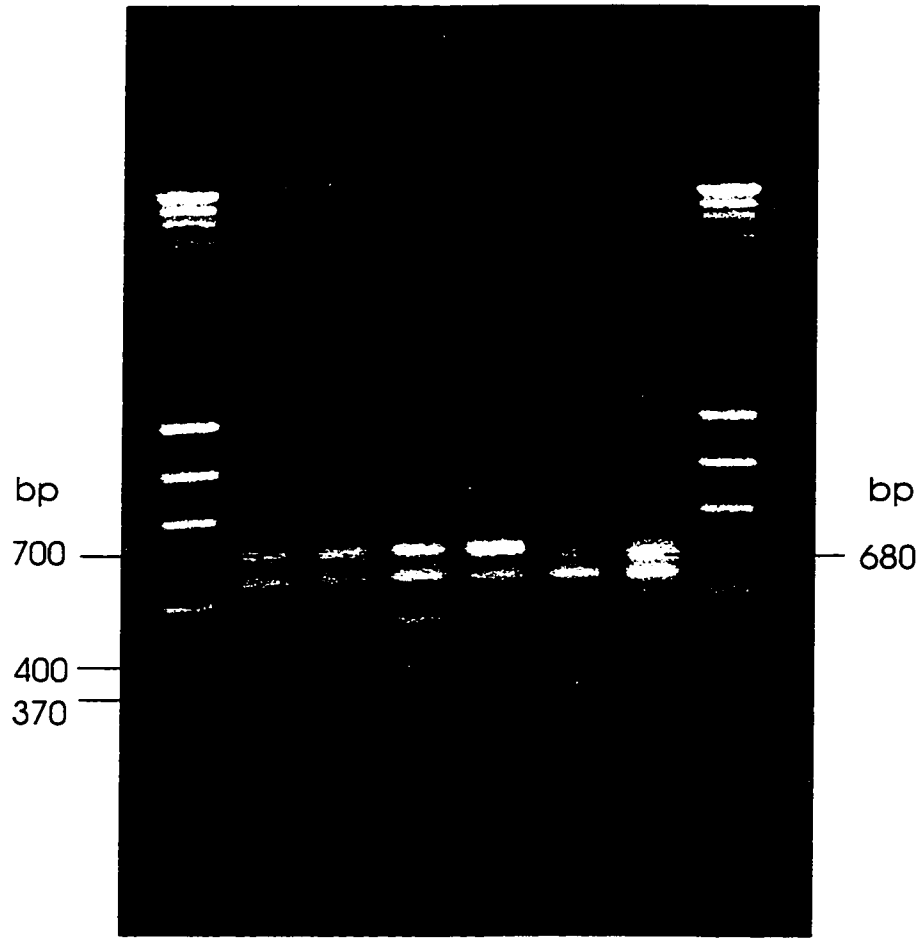
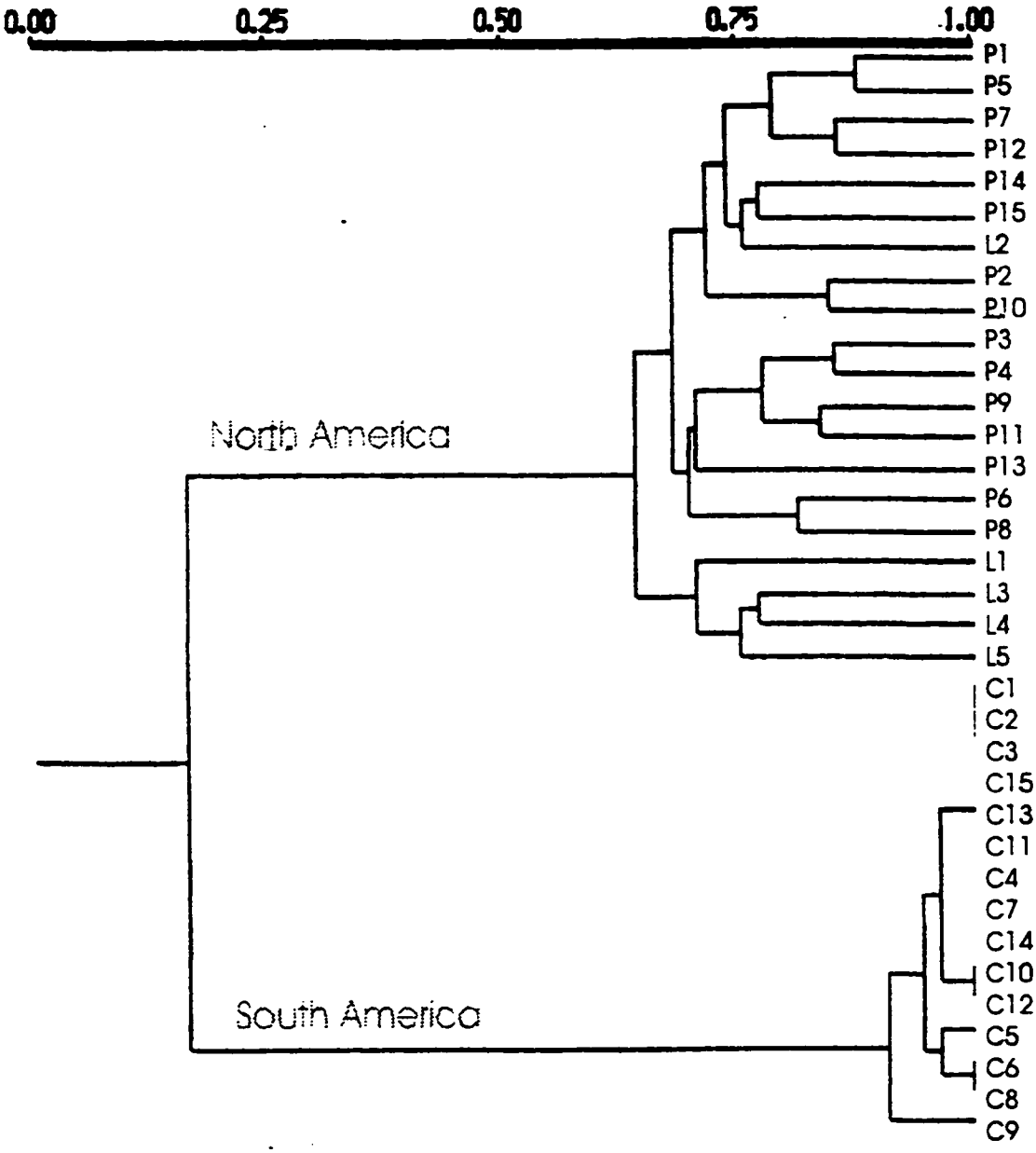

bp
700
400
370
bp
680

Figure 10. Phenogram portraying UPGMA clustering of 35 plants of *Fragaria* including five referable to North American ssp. *lucida* (L), 15 referable to North American ssp. *pacifica* (P) and 15 referable to south American ssp. *chiloensis* (C) based on Jaccard's coefficient derived from presence-absence data of band fragments from 12 primers.


0.00
0.25
0.50
0.75
1.00
P1
P5
P7
P12
P14
P15
L2
P2
P10
P3
P4
P9
P11
P13
P6
P8
L1
L3
L4
L5
C1
C2
C3
C15
C13
C11
C4
C7
C14
C10
C12
C5
C6
C8
C9
North America
South America

4.4.) Discussion:

Although morphological studies have suggested substantial differences between the North and South American subspecies of *F. chiloensis* (Chapter 2), this is the first molecular evidence for major genetic differentiation. This work suppports the current taxonomic separation of the North American and the South American plants with subspecies rank since they possess a high degree of between group genetic variation, and possess a number of morphological attributes that have a geographical basis but are sufficiently closely related within the genus as a whole to warrant subspecies rank (Crins *et al.* 1977, Semple 1974, 1992, Semple *et* al. 1988).

Since the South American subspecies is the major source for our cultivated crop, the fact that the North American wild strawberries are very different suggests an opportunity for incorporation of novel genetic variation in new cultivars. In addition, North American wild germplasm may be a valuable source for cold or stress tolerance and pest resistance since it is already naturally adapted to north temperate environmental conditions and pests. Incorporation of this material in new crops could lead to a very beneficial broadening of the genetic base of the commercial crop. The protection and further characterization of wild North American strawberry germplasm is, therefore, very worthwhile and the substantial efforts of the Canadian Clonal Genebank to protect and preserve wild North American germplasm are strongly supported.

Although partial genetic separation of the two North American subspecies is apparent, the extent of genetic differentiation is very limited in comparison to the differentiation found between the North and South American subspecies, thus supporting earlier morphological analyses (Chapter 2). Although some authors have considered the single character difference (hair type) to be adequate to recognize ssp. *pacifica* at this higher taxonomic level (Staudt 1989), the limited differentiation

of the two North American subspecies and the scale of difference suggests that their present taxonomic rank of subspecies is inappropriate . "Forma" has been described in summary as "distinctive phenotypes" (Cronquist 1988) that may (Carnevali & Ramirez 1990, Killeen 1990) or may not (Riesberg & Brouillet 1994) comprise distinct populations. Since the morphological character of hair type is consistent and some geographical separation is present, it is acceptable to maintain some distinction of these groups. However, there was little difference between the amount of between and within group genetic variation observed for the North American plants to warrant subspecies rank. In result, the recognition of the North American *F. chiloensis* ssp. *pacifica* taxa at the lower taxonomic rank of "forma" becomes more appropriate:

> *Fragaria chiloensis* (L.) Duchesne ssp. *lucida* (E. Vilm) f. *pacifica* (Staudt) Catling & Porebski, stat. et comb. nov. BASIONYM: *Fragaria chiloensis* ssp. *pacifica* Staudt, Canad. J. of Bot. 40: 869-886. 1962. Type: Ruby Beach, Olympic National Park, Jefferson Co., Washington, USA.: Locally common on scattered rock outcrops on seacoast. 30 July, 1959, G. Staudt & R.L. Taylor 88 (Holotype: DAO).

The high within group variation observed in comparison to that shown within the South American plants and morphological variation seen in hair orientaion, may prove to be useful germplasm for crop improvement. Relatively greater genetic variation suggested within the North American plants could be a result of either inherent variability or introgression involving another species of *Fragaria*, particularly the closely related *F. virginiana* Duchesne as recently suggested by Harrison *et al.* (1997).

CHAPTER 5: The clarification of taxonomic ranks based on the examination of the relationships of the Hawaiian *F.chiloensis* ssp. *sandwicensis* to the North American continental subspecies.

5.1.) Introduction:

Native strawberry plants from the Hawaiian Islands, once known as the Sandwich Islands, have been inconsistently classified as a variety (St. John 1961), distinct species (Vilmorin 1906), or have been placed in synonomy with the continental *F. chiloensis* (Hitchcock & Cronquist 1973). Based on the hypothesis that the Hawaiian *F. chiloensis* ssp. *sandwicensis* is distinct from the continental *F. chiloensis* and warrants subspecies rank (Ch.2.), the objective is to establish the relationship between the subspecies *sandwicensis* and the continental subspecies using RAPDs, and then to define its appropriate taxonomic rank.

5.2.) Methods:

The analysis included 54 plants. Thirty-five of the plants were included from North and South America as described in Chapter 4 with the inclusion of 19 additional plants from Maui. Two collecting trips were arranged and coordinated through Tim Flynn of the Tropical Botanical Garden in Laui, Hawaii. From the first collecting expedition, 10 plants were collected from the Kilohana quadrant central Maui in mid April 1997 by Arthur Medeiros of the Pacific Islands Science Center, Makawao, from gullies east of Pu'u Nianaiau at elevations of 6800 ft. Each plant was several meters apart and considered to be a separate population. From the second collecting trip, 9 plants were

obtained from the Kilohana quadrant approximately 20 km southeast of the previous collection site from the western slope of the Haleakala (6900 ft.) on July 30, 1997 by Chuck Chimera from an area of approximately 1 acre (4047m^2) within a subalpine shrubland/alien conifer forest. All plants were separated by several meters.

Plants were grown in eight inch pots in the greenhouse at the Central Experimental Farm, Ottawa (Plate I, II, III, appendix). DNA extractions from fresh leaf tissue and the RAPDs were carried out as described in Chapter 3. One hundred primers from UBC set #8 were screened. Twelve primers were selected which revealed adequate polymorphism within and between the three subspecies.

Replicates of two to three for all plants were repeated for each primer to check consistency. Band fragments that possessed within or between group variation were scored as present (1) or absent (0) and analysed using UPGMA clustering of Jaccard's coefficients (NTSYS ver. 1.70, Rohlf 1992).

5.3) Results:

From the 12 primers selected, all produced banding patterns that were 100% reproducible for the 2 or 3 replicates. All twelve primers produced banding patterns that were informative to detect polymorphism within the different site locations: UBC743 (5'CCACCCACAC), UBC746 (5'GGGTGTTGGG), UBC748 (5'CCCTTCTCCC), UBC756 (5'CCCTCCTCCT), UBC759 (5'CCAACCCACC), UBC772 (5'CCCACCACCC), UBC785 (5'CACCCAACCA), UBC790 (5'GGGTGTGGTT), UBC792 (5'CAACCCACAC), UBC795 (5'TGGTGTGGGT), UBC796 (5'AGAGGGAGGA) and UBC798 (5'GAGAGGAAGG). The size of amplification products varied

from 230 to 1500 bp in the 68 fragments scored. Most polymorphism in Hawaiian plants was present in fragments ranging from 240 to 980 bp. The number of band fragments for each primer varied from 1 to 13 polymorphic bands.

From the total of 68 polymorphic bands (see appendix), 24 band fragments were found in the Hawaiian plants. Three bands from three primers were unique to the Hawaiian plants including UBC743 (920, bp.), UBC772 (980 bp) and UBC790 (240 bp.) while three bands were absent from the Hawaiian plants which were present in both the North American and South American subspecies UBC790 (240 bp.), UBC796 (680 bp.) and UBC798 (310 bp.). While 15 bands were shared with the South American plants UBC743 (290 bp.), UBC759 (1450, 680 bp.), UBC772 (580, 410 bp.), UBC790 (520, 480 bp.) UBC792 (740 bp.), UBC795 (680 bp.), UBC796 (680 bp.), UBC798 (600, 540,370, 340 bp.) only 6 band fragments were common between the Hawaiian and North American subspecies including UBC743 (250 bp.) UBC748 (780, 740 bp.), UBC756 (700 bp.), UBC785 (1000 bp.), UBC795 (720 bp.). One example showing separation of the Hawaiian plants can be seen in Figure 11. In this case, a 920 bp. is unique in Hawaiian plants, a 320 bp. fragment is unique to the North American plants while a 700 bp. fragment is unique in South American plants.

The phenogram based on UPGMA clustering of Jaccard's coefficients revealed a clear division between the Hawaiian and North American and South American plants and was a reliable representation of relationships suggested by the Jaccard matrix (cophenetic correlation, r=0.97) (Fig. 12). The Hawaiian plants were shown to be more closely related to the South American ssp. *chiloensis* than the North American plants. In contrast to North American plants, within group variation among the Hawaiian plants was minimal and plants appeared to be clonal. Despite this, the significant distinctness indicates substantial, potential to contribute to a new strawberry crop,

but proportionately less protection is required than if they had proven to be a variable group like the North American and South American subspecies.

Figure 11. PCR amplimers on a 1.5% agarose gel with primer UBC8-743 (left to right): λ+ϕ marker (λ digested with Hind III +ϕX174 digested with Hae III), ssp. *pacifica* (P1), ssp.. *lucida* (L1), ssp. *chiloensis* (C1, C2), ssp. *sandwicensis* (S1, S2), λ+ϕ marker.

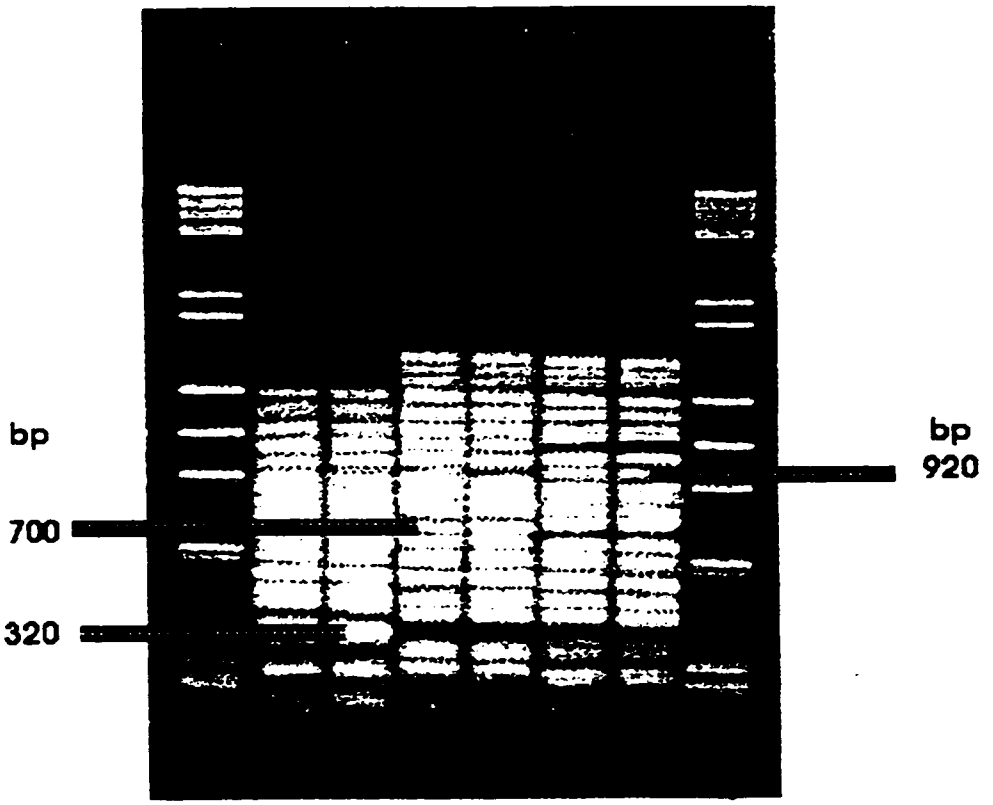
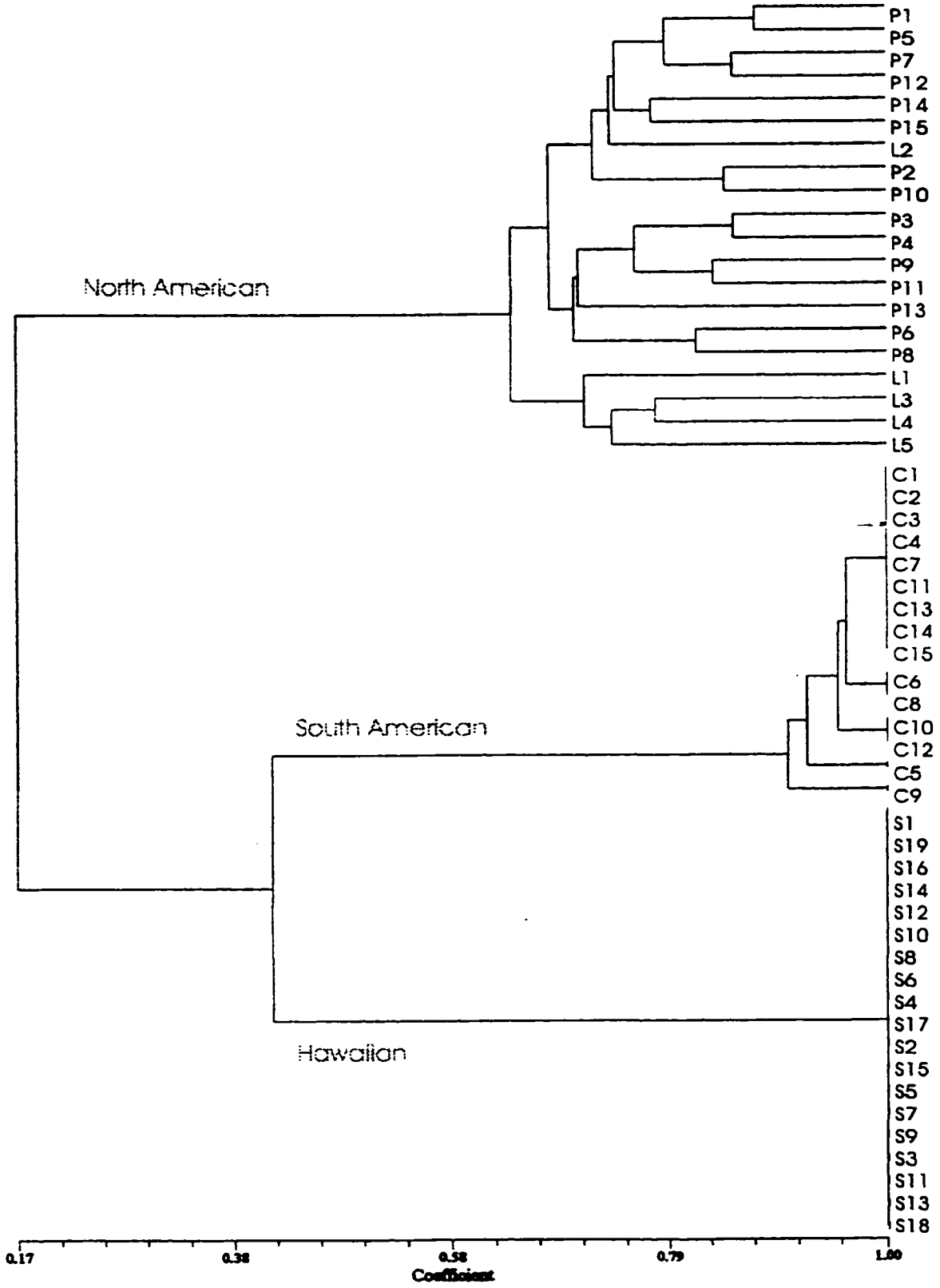

bp
700
320
bp
920

Figure 12. Phenogram portraying UPGMA clustering of 54 plants of *Fragaria* including five referable to North American ssp. *lucida* (L), 15 referable to North American ssp. *pacifica* (P) and 15 referable to south American ssp. *chiloensis* (C) and 19 from Maui, ssp. *sandwicensis* (S), based on Jaccard's coefficient derived from presence-absence data of band fragments from 12 primers.


P1
P5
P7
P12
P14
P15
L2
P2
P10
P3
P4
P9
P11
P13
P6
P8
L1
L3
L4
L5
C1
C2
C3
C4
C7
C11
C13
C14
C15
C6
C8
C10
C12
C5
C9
S1
S19
S16
S14
S12
S10
S8
S6
S4
S17
S2
S15
S5
S7
S9
S3
S11
S13
S18
North American
South American
Hawaiian
0.17
0.38
0.58
0.79
1.00
Coefficient

5.4) Discussion:

Hawaiian plants were shown to be distinctive displaying high between-group variation thus supporting the status of rank of subspecies as suggested by Staudt (1989). Although there was clear between-group variation illustrated, no within-group variation was observed in contrast to all other subspecies groups examined. The lack of variation observed within the Hawaiian plants may be a result of insufficient time to undergo genetic divergence and/or simply a result of the small geographical sampling range of Maui. Sample size may also provide an explanation since representation from the Island of Hawaii was not obtained. However, a fair representation of Maui was provided over a 20 km range providing representaion of approximately 70% of the available population area and thus, suggests that sample size may not be the only cause of lack of variation.

If all Hawaiian plants collected are clones as suggested by the sample in Maui, they are still sufficiently different from the South American ssp. *chiloensis* and are potentially useful in broadening the genetic base of our cultivated crop through the introduction of new and promising varaint DNA.

CHAPTER 6: GENERAL CONCLUSIONS:

6.1.Germplasm Preservation:

The primary objective of this thesis was to examine the pattern of variation within *F.chiloensis*, develop a classification that was both operational and an accurate portrayal of genetic relationships in order to provide efficient protection and utilization of genetic variation for use in crop improvement. When the pattern of variation was examined within and between the various subspecies, North American plants which exhibit interesting patterns of variation included plants from areas such as South of Hazard Point (P6), Campania Island (P8), Smith Point (L3, L4), the second westerly Island of Emily Island (L1) and south of Kimura Lake (L5). Plants from South America requiring good representation due to higher variation included the plants examined from Island of Lemuy (C6, C8), Termas de Chillan (C9, C10), Camahueto River (C12), and Cohaique (C5). Regardless of whether these taxonomic ranks are accepted in this study by all authors, the relationship between the groups have been established for the first time through the analysis of quantitative data. For instance, if the three geographic groups had proven to be more or less identical, there would be little reason to consider the North American and Hawaiian plants to be valuable in crop improvement. However, since they are very different from the South American progenitor, it indicates that they have potential to produce very new and different strawberry crops while simultaneously retaining desirable characteristics of the present crop. The finding that geographic established subspecies were quite distinct requires that material representative of the genetic variation within each be protected. The amount of divergence and within-group genetic variation suggests that up to 60% of *F. chiloensis* subspecies germplasm to be protected should be

North American including both f. *pacifica* and f. *lucida*. Since all of the Hawaiian plants studied represent a single clone, only 1 to 2 % of the protected germplasm should be from Maui and the remaining 38% should constitute South American plant populations of ssp. *chiloensis*.

6.2. Taxonomy:

This research has lead to the clarification of three geographic groups including North American, South American and Hawaiian plants. These groups are recognized at the subspecies level based on the North American concept of 'subspecies' being characterized by both morphological distinctness and by allopatric distribution. Since two putative subspecies within North America did not meet this criteria, their classification was recommended at the lower taxonomic rank of 'forma'. By providing such a refined and relatively well supported classification system, the foundation for germplasm protection is achieved. Without accurate taxonomic representation, valuable germplasm could be lost and/or redundant material kept unnecessarily. Plants representing higher taxonomic levels such as subspecies, should warrant greater representation than plants from lower taxonomic levels like "forma". Systematics provides a building block for many different kinds of research. Without proper identification of any study group, research can become meaningless due to a lack of both proper association and reproducibility.

6.3. Limitations and Future Research:

As shown here, the RAPD technique has been shown to be quite useful with regards to

assessing a pattern of variation and relationship. This procedure in combination with morphology and geography has provided a powerful and cost effective means to aid in germplasm protection and utilization. Combining several techniques and utilizing all information available appears to be the most efficient means of approaching any systematic work.

In this study, the primary motivation was to assess the pattern of variation to aid in germplasm protection and utilization, not to examine the evolution of *Fragaria*. Further research could include other species of *Fragaria* in order to answer evolutionary questions of phylogenetic relationships. For example, it may be interesting to compare the evolutionary relationship of the octoploid unique to the Island of Iturup in southern Kuriles with the North American, South American and Hawaiian octoploids using a diploid as the outgroup. When comparing higher taxonomic levels such as the species and family level, another technique such as DNA sequencing may be more appropriate or useful in addition to or instead of RAPDs provided the selected genes are appropriate sizes or evolve at a rate which can aid in establishing evolutionary patterns. When DNA sequencing fails to obtain conclusive results as, for example, within the family Rosaceae, RAPDs may help to clarify some of the unresolved phylogenies (Potter *et al.* 1997).

Although this analysis enables a determination of the relative proportion of germplasm to be protected on the basis of genetic variation and relationship, a more reliable assessment could be made with a larger sample size of all plants and particularly an inclusion of plants from Hawaii in addition to Maui to provide a more accurate assessment of ssp. *sandwicensis*. In addition, the extent of introgressive hybridization involving *F. virginiana* in the North American sample, would have to be established in order to prevent over-representation of North American plants due to protection of genes of *F. virginiana* within introgressed *F. chiloensis*. It is to be noted however, that the

natural hybrids may prove very useful in deriving new crops since they have already undergone some environmental selection thus could potentially save many steps in a breeding program due to their natural genetic stability (Dale 1994).

6.4. Final Comments:

This research, indicating the genetic divergence of geographic subspecies, directly supports the substantial effort of the Canadian Clonal Genebank to protect variation of the North American plants from the pacific coast of Canada. In addition, with strawberries currently worth at least 60 million in annual farmgate in Canada and with genetically diverse and unique Canadian germplasm available to improve the genetically depauperate crop, strawberries appear to be in a good position to assume a more important role in Canadian Agriculture.

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APPENDIX A: The output of the discriminant analyses with misclassification matrices obtained from SAS.

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95 Observations 94 DF Total
10 Variables 91 DF Within Classes
4 Classes 3 DF Between Classes

Class Level Information

TYPE	Frequency	Weight	Prior Proportion	Probability
1	30	30.0000	0.315789	0.250000
2	20	20.0000	0.210526	0.250000
3	27	27.0000	0.284211	0.250000
4	18	18.0000	0.189474	0.250000

The SAS System 15:08 Friday, May 15, 1998 11

Discriminant Analysis Pooled Covariance Matrix Information

Covariance Matrix Rank	Natural Log of the Determinant of the Covariance Matrix
10	10.004904

The SAS System 15:08 Friday, May 15, 1998 12

Discriminant Analysis Linear Discriminant Function

$$\text{Constant}_j = -.5\ \bar{X}_j' \text{COV}^{-1} \bar{X}_j \qquad \text{Coefficient Vector}_j = \text{COV}^{-1} \bar{X}_j$$

	TYPE 1	2	3	4
CONSTANT	-38.54570	-46.28079	-47.47074	-75.35683
C1	0.28069	0.26307	0.31950	0.88644
C2	0.89319	1.18155	0.94999	0.54598

C3	0.36654	0.33499	0.38583	0.64032
C4	-1.30637	-1.82964	-1.85857	-1.57793
C5	0.32471	0.44169	0.60801	-0.02404
C6	0.67804	0.72943	0.82089	0.76239
C7	16.47405	17.79327	17.91765	26.46853
C8	5.46008	6.81662	6.46817	8.23708
C9	1.65982	1.82149	1.96987	1.59582
C10	-0.30409	-0.08499	-0.12671	0.32818

Cross-validation Summary using Linear Discriminant Function

Generalized Squared Distance Function: Posterior Probability of Membership in each TYPE:

$$D_j^2(X) = (X-\bar{X}_{(X)j})' \, \bar{COV}_{(X)}^{-1} \, (X-\bar{X}_{(X)j}) \qquad Pr(j|X) = \exp(-.5\, D_j^2(X)) \,/\, SUM_k \exp(-.5\, D_k^2(X))$$

Number of Observations and Percent Classified into TYPE:

From TYPE	1	2	3	4	Total
1	15	7	7	1	30
	50.00	23.33	23.33	3.33	100.00
2	6	5	7	2	20
	30.00	25.00	35.00	10.00	100.00
3	7	7	13	0	27
	25.93	25.93	48.15	0.00	100.00
4	0	2	0	16	18
	0.00	11.11	0.00	88.89	100.00
Total	28	21	27	19	95
Percent	29.47	22.11	28.42	20.00	100.00
Priors	0.2500	0.2500	0.2500	0.2500	

Error Count Estimates for TYPE:

1	2	3	4	Total

Rate	0.5000	0.7500	0.5185	0.1111	0.4699
Priors	0.2500	0.2500	0.2500	0.2500	

***repeated joining North American groups:**

Discriminant Analysis

95 Observations 94 DF Total
10 Variables 92 DF Within Classes
3 Classes 2 DF Between Classes

Class Level Information

TYPE	Frequency	Weight	Prior Proportion	Probability
1	50	50.0000	0.526316	0.333333
3	27	27.0000	0.284211	0.333333
4	18	18.0000	0.189474	0.333333

Discriminant Analysis Pooled Covariance Matrix Information

Covariance Matrix Rank	Natural Log of the Determinant of the Covariance Matrix
10	10.10441

Discriminant Analysis Linear Discriminant Function

$$\text{Constant}_j = -.5\,\bar{X}_j' \text{COV}^{-1} \bar{X}_j \qquad \text{Coefficient Vector}_j = \text{COV}^{-1} \bar{X}_j$$

	TYPE 1	TYPE 3	TYPE 4
CONSTANT	-38.79265	-43.86257	-70.51477

C1	0.29107	0.33968	0.91570
C2	0.78379	0.68784	0.23258
C3	0.38361	0.41990	0.68230
C4	-1.10438	-1.38433	-1.01566
C5	0.27992	0.50411	-0.15388
C6	0.66425	0.78133	0.71385
C7	16.10965	16.86745	25.29811
C8	4.95922	5.25687	6.82497
C9	1.61121	1.83869	1.43428
C10	-0.39802	-0.33522	0.08909

Cross-validation Summary using Linear Discriminant Function

Generalized Squared Distance Function: Posterior Probability of Membership in each TYPE:

$$D^2_j(X) = (X-\bar{X}_{(X)j})' \, COV^{-1}_{(X)} \, (X-\bar{X}_{(X)j}) \qquad Pr(j|X) = \exp(-.5\, D^2_j(X)) \,/\, SUM_k \exp(-.5\, D^2_k(X))$$

Number of Observations and Percent Classified into TYPE:

From TYPE	1	3	4	Total
1	29	17	4	50
	58.00	34.00	8.00	100.00
3	11	16	0	27
	40.74	59.26	0.00	100.00
4	1	0	17	18
	5.56	0.00	94.44	100.00
Total	41	33	21	95
Percent	43.16	34.74	22.11	100.00
Priors	0.3333	0.3333	0.3333	

Error Count Estimates for TYPE:

1	3	4	Total

Rate	0.4200	0.4074	0.0556	0.2943
Priors	0.3333	0.3333	0.3333	

APPENDIX B: Covariance matrix obtained from the linear discrimination of the standarized data using SGplus 7.0.

Discriminant Analysis for MORPHO.TYPE

Within-Group Covariance Matrix

	MORPHO.C1	MORPHO.C2	MORPHO.C3	MORPHO.C4
MORPHO.C1	58.2211	41.7090	22.7200	37.3754
MORPHO.C2	41.7090	37.0977	21.5448	34.2426
MORPHO.C3	22.7200	21.5448	16.2873	21.7501
MORPHO.C4	37.3754	34.2426	21.7501	33.8944
MORPHO.C5	36.3347	31.5934	17.4963	28.5498
MORPHO.C9	-12.4949	-10.8906	-5.57708	-8.80862
MORPHO.C11	-0.29735	-0.08679	-0.09622	-0.07052
MORPHO.C12	2.85081	2.25972	1.65278	2.34736
MORPHO.C13	1.83094	1.73356	1.30250	1.92243
MORPHO.C18	8.95764	6.74083	3.60037	6.12882
MORPHO.C40	-12.8140	-10.5742	-7.74176	-10.5932
MORPHO.C42	0.20192	0.08065	0.08140	0.09129
MORPHO.RATC1AC49	0.40114	0.40102	0.37722	0.47644

Within-Group Correlation Matrix

	MORPHO.C1	MORPHO.C2	MORPHO.C3	MORPHO.C4
MORPHO.C1	1.00000	0.89746	0.73781	0.84136
MORPHO.C2	0.89746	1.00000	0.87648	0.96567
MORPHO.C3	0.73781	0.87648	1.00000	0.92570
MORPHO.C4	0.84136	0.96567	0.92570	1.00000
MORPHO.C5	0.87353	0.95152	0.79527	0.89957
MORPHO.C9	-0.24943	-0.27235	-0.21049	-0.23046
MORPHO.C11	-0.13117	-0.04796	-0.08025	-0.04077
MORPHO.C12	0.43083	0.42782	0.47225	0.46494
MORPHO.C13	0.24049	0.28525	0.32346	0.33094
MORPHO.C18	0.53440	0.50379	0.40610	0.47921
MORPHO.C40	-0.24865	-0.25705	-0.28403	-0.26941
MORPHO.C42	0.09295	0.04651	0.07084	0.05508
MORPHO.RATC1AC49	0.13617	0.17053	0.24209	0.21196

	MORPHO.C5	MORPHO.C9	MORPHO.C11	MORPHO.C12
MORPHO.C1	36.3347	-12.4949	-0.29735	2.85081
MORPHO.C2	31.5934	-10.8906	-0.08679	2.25972
MORPHO.C3	17.4963	-5.57708	-0.09622	1.65278
MORPHO.C4	28.5498	-8.80862	-0.07052	2.34736
MORPHO.C5	29.7174	-9.28699	-0.03523	1.71430
MORPHO.C9	-9.28699	43.1014	0.17272	-0.79974
MORPHO.C11	-0.03523	0.17272	0.08827	-0.03984
MORPHO.C12	1.71430	-0.79974	-0.03984	0.75204
MORPHO.C13	1.10279	-0.33264	0.02044	0.38873
MORPHO.C18	5.96506	-1.21468	-0.06214	0.33107
MORPHO.C40	-9.31013	12.9581	-0.18842	0.03443
MORPHO.C42	0.08938	0.00773	0.00543	0.00356
MORPHO.RATC1AC49	0.18760	-0.29398	-0.00378	0.05478

	MORPHO.C5	MORPHO.C9	MORPHO.C11	MORPHO.C12
MORPHO.C1	0.87353	-0.24943	-0.13117	0.43083
MORPHO.C2	0.95152	-0.27235	-0.04796	0.42782
MORPHO.C3	0.79527	-0.21049	-0.08025	0.47225
MORPHO.C4	0.89957	-0.23046	-0.04077	0.46494
MORPHO.C5	1.00000	-0.25949	-0.02175	0.36263
MORPHO.C9	-0.25949	1.00000	0.08855	-0.14047
MORPHO.C11	-0.02175	0.08855	1.00000	-0.15462
MORPHO.C12	0.36263	-0.14047	-0.15462	1.00000
MORPHO.C13	0.20275	-0.05078	0.06895	0.44925
MORPHO.C18	0.49810	-0.08422	-0.09520	0.17379
MORPHO.C40	-0.25287	0.29224	-0.09390	0.00588
MORPHO.C42	0.05759	0.00413	0.06424	0.01443
MORPHO.RATC1AC49	0.08913	-0.11598	-0.03299	0.16361

	MORPHO.C13	MORPHO.C18	MORPHO.C40	MORPHO.C42
MORPHO.C1	1.83094	8.95764	-12.8140	0.20192
MORPHO.C2	1.73356	6.74083	-10.5742	0.08065
MORPHO.C3	1.30250	3.60037	-7.74176	0.08140
MORPHO.C4	1.92243	6.12882	-10.5932	0.09129
MORPHO.C5	1.10279	5.96506	-9.31013	0.08938

MORPHO.C9	-0.33264	-1.21468	12.9581	0.00773
MORPHO.C11	0.02044	-0.06214	-0.18842	0.00543
MORPHO.C12	0.38873	0.33107	0.03443	0.00356
MORPHO.C13	0.99556	0.23863	-0.66996	-0.00104
MORPHO.C18	0.23863	4.82592	-3.58741	0.01852
MORPHO.C40	-0.66996	-3.58741	45.6159	-0.18191
MORPHO.C42	-0.00104	0.01852	-0.18191	0.08105
MORPHO.RATC1AC49	0.15968	-0.01868	-0.55923	0.00145

	MORPHO.C13	MORPHO.C18	MORPHO.C40	MORPHO.C42
MORPHO.C1	0.24049	0.53440	-0.24865	0.09295
MORPHO.C2	0.28525	0.50379	-0.25705	0.04651
MORPHO.C3	0.32346	0.40610	-0.28403	0.07084
MORPHO.C4	0.33094	0.47921	-0.26941	0.05508
MORPHO.C5	0.20275	0.49810	-0.25287	0.05759
MORPHO.C9	-0.05078	-0.08422	0.29224	0.00413
MORPHO.C11	0.06895	-0.09520	-0.09390	0.06424
MORPHO.C12	0.44925	0.17379	0.00588	0.01443
MORPHO.C13	1.00000	0.10887	-0.09942	-0.00368
MORPHO.C18	0.10887	1.00000	-0.24179	0.02962
MORPHO.C40	-0.09942	-0.24179	1.00000	-0.09461
MORPHO.C42	-0.00368	0.02962	-0.09461	1.00000
MORPHO.RATC1AC49	0.41449	-0.02202	-0.21446	0.01323

	MORPHO.RATC1AC49
MORPHO.C1	0.40114
MORPHO.C2	0.40102
MORPHO.C3	0.37722
MORPHO.C4	0.47644
MORPHO.C5	0.18760
MORPHO.C9	-0.29398
MORPHO.C11	-0.00378
MORPHO.C12	0.05478
MORPHO.C13	0.15968
MORPHO.C18	-0.01868
MORPHO.C40	-0.55923

MORPHO.C42	0.00145
MORPHO.RATC1AC49	0.14907

	MORPHO.RATC1AC49
MORPHO.C1	0.13617
MORPHO.C2	0.17053
MORPHO.C3	0.24209
MORPHO.C4	0.21196
MORPHO.C5	0.08913
MORPHO.C9	-0.11598
MORPHO.C11	-0.03299
MORPHO.C12	0.16361
MORPHO.C13	0.41449
MORPHO.C18	-0.02202
MORPHO.C40	-0.21446
MORPHO.C42	0.01323
MORPHO.RATC1AC49	1.00000

APPENDIX C: Primer screening with UBC set #8. Asterix indicates selected primer.

PRIMER	AMPLIFICATION	POLYMORPHISM	BACKGROUND
701	YES	NO	
702	YES	NO	
703	NO		
704	YES	NO	
705	YES	YES (LITTLE)	YES
706	NO		
707	YES	NO	
708	YES	YES (LITTLE)	YES
709	NO		
710	YES	YES (LITTLE)	YES
711	YES	YES (LITTLE)	YES
712	YES	YES	YES
713	NO		
714	YES	NO	YES
715	N/A		
716	YES	YES (LITTLE)	YES
717	YES	YES (LITTLE)	YES
718	YES	YES (LITTLE)	YES
719	N/A		
720	NO		
721	YES	NO	
722	NO		
723	NO		
724	NO		

PRIMER	AMPLIFICATION	POLYMORPHISM	BACKGROUND
725	YES	YES (LITTLE)	YES
726	YES	NO	YES
727	YES	NO	YES
728	NO		
729	YES	YES (LITTLE)	YES
*730	YES	YES	
731	YES	YES (LITTLE)	YES
732	YES	YES (LITTLE)	YES
733	YES	NO	
734	YES	YES (LITTLE)	YES
735	NO		
736	YES	NO	
737	NO		
738	YES	NO	YES
739	YES	NO	YES
740	YES	NO	YES
741	NO		
742	NO		
*743	YES	YES	
744	YES	YES (LITTLE)	
745	YES	YES (LITTLE)	YES
*746	YES	YES	
747	YES	YES (LITTLE)	YES
*748	YES	YES	
749	YES	YES (LITTLE)	YES
750	YES	YES (LITTLE)	YES

PRIMER	AMPLIFICATION	POLYMORPHISM	BACKGROUND
*751	YES	YES	
752	YES (FAINT)		
753	N/A		
754	YES	YES (LITTLE)	YES
755	N/A		
*756	YES	YES	
757	N/A		
758	YES	NO	
*759	YES	YES	
*760	YES	YES	
761	NO		
762	NO		
763	NO		
764	NO		
765	YES		YES
766	YES		YES
767	YES		YES
768	NO		
769	YES		YES
770	YES	NO	YES
771	YES (FAINT)	NO	
772			
773			
774			
775	YES	NO	YES
776			

PRIMER	AMPLIFICATION	POLYMORPHISM	BACKGROUND
777	YES		YES
778			
779			
780			
781			
782			
783			
784			
*785	YES	YES	
786			
787			
788	NO		
789			
*790	YES	YES	SOME
791			
792			
793			
794			
795			
796			
797			
798			
799			
800	YES		YES

APPENDIX D: Collections and collection numbers (or dates) of 95 specimens utilized in Chapter 2.

BA: 6053, Groudona, 23/8/1972; 66220, Groudona, 23/8/1972; 49834, Groudona, 23/8/1972; 46082, Groudona, 23/8/1972; 28333, Groudona, 23/8/1972: 40673, Groudona, 23/8/1972;
BAB: 5235, Boelcke & Correa, 5/1/1952; 10816, Boelcke *et al.*, 15/1/1964; 2093, Gomez *et al.*, 15/1/1984; 56043, Spegazzini, 25/3/1934;
BP: 64377, Degener 12514; 64398, Forbes, 9/6/1915; 64394, Forbes 145; Forbes 165; 487441, Gillett & Carlson 1739; 429232, Harrison, 30/6/1973; 64397, Herbst *et al.* 902; 36061, Herbst *et al.* 3492; 416903, Higashino 770; 64383, Skottsberg 602; 64385, Rock 3722; 64392, Rock 3724; 64389, 492458, Rock; 64384, Rock 3752; 468105, Warshauer & McEldowney 2665;
DAO: 397172, Calder *et al.* 31151; 397194, Calder *et al.*34654; 397193, Calder *et al.*20927; 397191, Calder *et al.* 21106; 397167, Calder *et al.*, 29125; 397177, 21232; 397174, Calder *et al.*34840; 397192, Calder *et al.*2099; 140813, Pinder-Moss 91; 397170, Ginns 22;
NY: 6432, Anderson, 11/5/1941; 211956, Barkley, 4/21/1956; 647, Bartlett & Grayson, 12/7/1952; 2745, Ceron & Ceron, 16/11/1987; 6843, Cronquist, 14/4/1953; 2, Funston, 27/5/1892; 7552, Gorman, 12/4/1926; 145, Heller, 8/6/1902; 29155, Howell, 25/4/1954; 94456, Hunnewell 19556; Linguer, 18/1/1934; 5219, Muller, 12/10/1982; 59, Oliver & Oliver, 7/1946; 18364, Wilkes, 0/0/1888;
OSC: 46172, Detling, 4/5/1948; 14166, Elmer 4799; 64572, Gilkey, 2/4/1947; 46239, Henderson 15568; 38351, Ireland 938; 46193, Sheldon 12251; 138395, Williams 2870;
SGO: 73563, Avevedo; 129345, Cameron *et al.*, 14/1/1990; 49801, Reev, 0/0/1959; 99501, Sparre 4453; 98621, Sparre 3198;
SI: 32918, Cabrera *et al.*, 8/12/1981;
UBC: 163534, Annas & Klinka, 21/5/1974; 135037, Beamish 610103; 124711, Calder *et al.* 21232; 124245, Calder & Taylor 34654; 107064, Corfman & Freyman 4; 3497, Eastham, 26/4/1939; 107671, Eastham, 6/5/1964; 33443, Eastham 8115; 60327, Pillsbury 392; 135773, Markham & Lambert 9; 49772, Marsdal, 8/1955; 204654, McCutcheon & Ellis V204654; 206607, McCutcheon, 15/6/1992;
UC/Jeps: 149341, Illin 153, 2/1903; 313176, Diem 2940; 636404, Morrison & Wagenknecht 17515; 924277, Reed 215; 313177, Staudt & Taylor 88; 52289, Summers, 17/2/1886; 198726, Tracy 1083; 1536909, True 616; 658544, Vargas 11070; 561447, West 4660; 562005, West 5116; 127975, Yates 3722;
US: 1176712, Buchtien , 30/9/1896; 2881083, Higashino 770, 6/1975; 1619415 MacMillan & Erlanson 2; 424, Mann & Brigham 424; 1093611, Skottsberg & Skottsberg, 3/12/1916;
WS: 82619, Hedrick 150; 1162, Lamb, 6/7/1897; 223503 Norbay 21/7/1936;

APPENDIX E: Summary sheet of RAPD protocol - A list of parameters that should be standardized in a RAPD-PCR reaction to allow cross-comparison of results from different RAPD profiling studies on *Fragaria*.

A. RAPD_PCR REACTION CHEMICALS

Reference for DNA extraction technique: Porebski *et al.* Plant Molecular Reporter, 1997

DNA template quantity:	**1-1.5 ng, 2-3μl** (3µl of 0.5 ng)
Concentration of $MgCl_2$ added:	**2mM, 2μl** (1µl)
Concentration of dNTPs added:	**0.1mM, 2μl** (1µl)
Concentration of primer added:	**0.4µM, 4μl** (2µl)
Taq polymerase brand and vol.:	**Stoffel fragment 2u, 0.2μl;** (1u, 0.1µl)
PCR 10x buffer brand and vol.:	**Perkin Elmer 2.5μl** (1.25µl)
Volume of dH_20:	**12.3-11.3μl** (4.15 µl)
Total volume of PCR reaction:	**25μl** (12.5µl)
Primer information:	**UBC set #8**

B. HARDWARE FOR RAPD-PCR

Make and model of thermocycler: **MJ Research Inc. PTC100 Thermocycler**

C. THERMOCYCLER TEMPERATURE PROFILES

Cycle programme:	**RAPD 13**
Initial denaturation step:	**94 °C for 15 sec.**
Cyclic denaturation step:	**same**
Cyclic annealing step:	**35 °C for 30 sec.**
Ramp rate between annealing and extension step(°C/s):	**Minumum**
Cyclic extension step:	**72 °C**
Total # of cyclic steps:	**30 cycles**
Final extension step:	**72 °C for 7 min.**
Temperature modifications:	**None**

D. ANALYSIS OF RAPD_PCR FRAGMENTS

Type of electrophoresis used (agarose etc.):	**Agarose**
Brand of media:	**SeaKem**
Concentration of gels used (%):	**1.5%**
Electrophoresis conditions:	**0.05 mA, 85 V, 1.5 Hr**
Chemical used to stain DNA:	**Ethidium bromide**
Type and recipe of electrophoresis buffer used:	**TBE**

E. RAPD FRAGMENT PRESENCE/ABSENCE TABLE- presence indicated by 1 and 0 absence - Genebank accession label, primer, fragment size in bps (see APPENDIX G).

APPENDIX F: Original data matrix of presence (1) or absence (0) for 62 polymorphic band fragments obtained from RAPD analysis generated using 12 primers with 35 plants including f. *pacifica* (P), f. *lucida* (L), and ssp. *chiloenis* (C) from Chapter 4.

1 62L 35L 0
743-870 743-840 743-720 743-700 743-680 743-330 743-320 743-290 743-260 743-250 743-240
743-230 746-720 748-780 748-740 756-1150 756-840 756-700 756-580 759-1500 743-800 748-720
759-1450 759-720 759-700 759-680 759-640 759-620 759-550 759-690 772-1000 772-580 772-540
772-410 785-1000 785-980 785-560 785-390 785-350 785-400 790-620 790-560 790-520 790-480
790-350 790-600 790-500 792-740 792-700 792-680 792-400 792-370 792-420 795-720 795-680
796-450 798-600 798-540 798-370 798-340 756-650 798-300
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14 P15 L1 L2 L3 L4 L5 C1 C2 C3 C4 C5 C6 C7 C8
C9 C10 C11 C12 C13 C14 C15
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1
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1 1 1 1 0 1 1 1 1 1 1 1 1 1 1 0 1 1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
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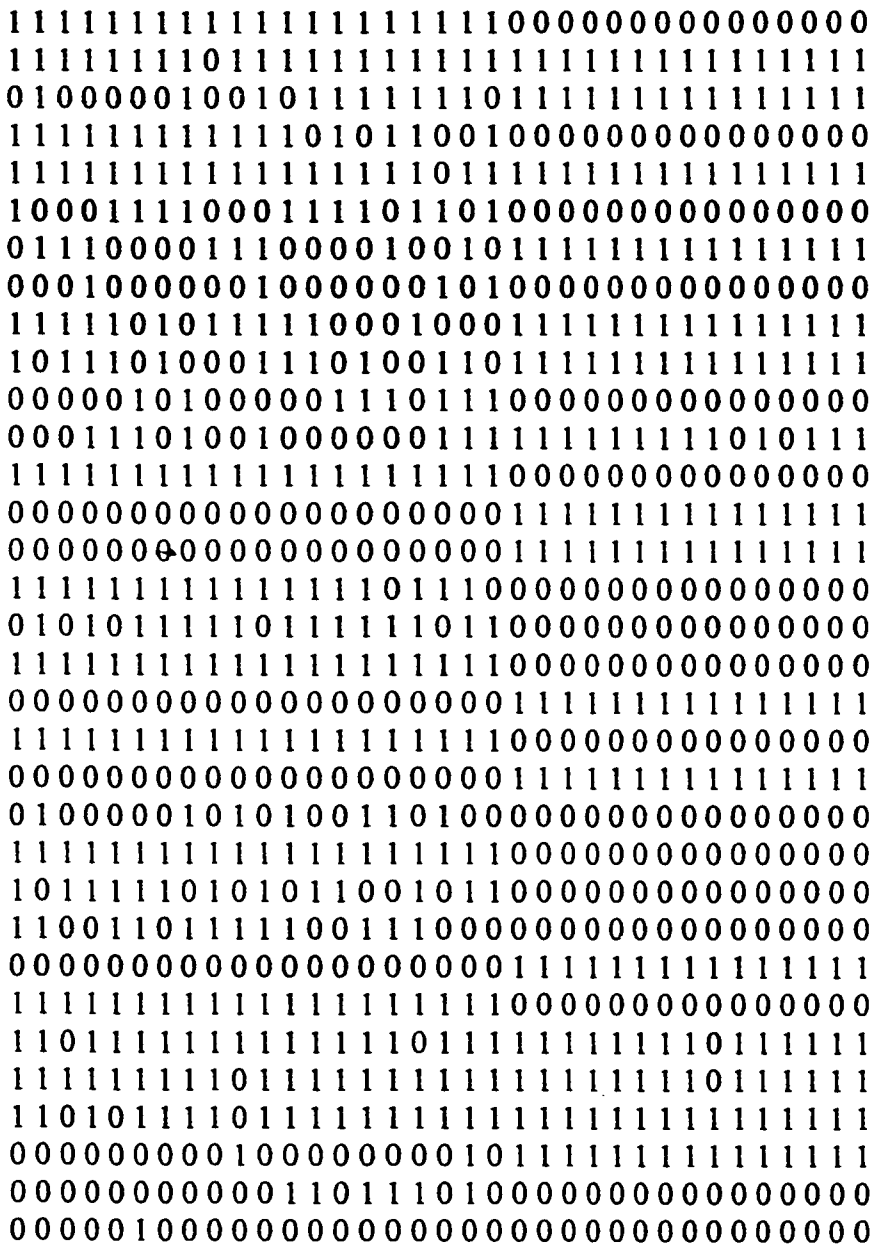

APPENDIX G: PCR products obtained exhibiting polymorphism using 62 primers with ssp. *pacifica*, spp. *lucida*, and ssp. *chiloensis* from Chapter 4 where (+) indicates band presence, (-) indicates band absence and (+/-) indicates within group variation.

Sequence	Band Fragment (bp)	ssp. *pacifica*	ssp. *lucida*	ssp. *chiloensis*
UBC743 (5'CCACCCACAC)	870	-	-	+
	840	+	+	-
	720	+	+	-
	700	-	-	+
	680	-	-/+	-
	330	-/+	-/+	-
	320	-/+	-	-
	290	-	-	+
	260	-	-	-/+
	250	-/+	-/+	-
	240	-/+	-/+	-
	230	-	-	-/+
UBC746 (5'GGGTGTTGGG)	720	+	+	-
UBC748 (5'CCCTTCTCCC)	780	-/+	-	+
	740	+	+	-
	720	+	+	-
UBC756 (5'CCCTCCTCCT)	1150	-/+	+	-
	840	-/+	+	-
	700	-/+	+	-
	650	-/+	-/+	-
	580	-/+	-	-
UBC759 (5'CCAACCCACC)	1500	+	+	-
	1450	-	-	+
	720	-	+	-
	700	+	+	-
	690	-	-	+
	680	-	-	+
	640	-/+	-/+	-
	620	-	+	-
	550	-/+	+	+

UBC772 (5'CCCACCACCC)	1000	-/+	-/+	+
	580	-/+	-/+	-
	540	+	-/+	+
	410	-/+	-/+	-
UBC785 (5'CACCCAACCA)	1000	-/+	-/+	+
	980	-/+	-/+	-
	560	-/+	-/+	+
	400	-/+	-/+	-
	390	-/+	-/+	+
	350	-/+	-/+	-/+
UBC790 (5'GGGTGTGGTT)	620	-/+	-/+	-
	600	-/+	-/+	-
	560	-	-	+
	520	-	-	+
	500	+	+	-
	480	+	-/+	-
	350	-	-	+
UBC792 (5'CAACCCACAC)	740	+	+	-
	700	-	-	+
	680	-/+	-/+	-
	420	-/+	-/+	-
	400	+	+	-
	370	-/+	-/+	-
UBC795 (5'TGGTGTGGGT)	720	-/+	-/+	-
	680	-	-	+
UBC796 (5'AGAGGGAGGA)	450	+	+	-
UBC798 (5'GAGAGGAAGG)	600	-/+	-/+	-/+
	560	-/+	-/+	-
	540	-/+	+	-/+
	370	-/+	+	+
	340	-/+	-/+	-
	300	-/+	-	-

APPENDIX H: The collection numbers, sites, locations with latitudes and longitudes for each plant used in the RAPD analysis.

Subspecies (OTU)	Coll. No.	Site	Location	Lat./Long.
P1	893	120	Comber's Beach, Vancouver Isl.	49°02'10"N, 125°41'30"W
P2	130	126	Sharp Point, Openit Penn., Refuse Cove, V.I.,	49°30'00"N, 126°15'30"W
P3	857	119	Head of Florencia Bay, V.I.	49°00'00"N, 125°39' 00"W
P4	957	121	Long Beach, V.I.	49°02'30"N, 125° 42'05"W
P5	1060	128	Small Isl., SW of Amos Isl., SW of Kyuquot, V.I.,	50°00'40"N, 127°20'30"W
P6	1094	132	SW of O-Ya-Kum-La Indian Res., S of Hazard Pt..	50°29'25"N, 128°01'05"W
P7	1178	143	Beach in Klaskino, SE of Ancorage Isl., V.I.	50°17'50"N, 127°48' 50"W
P8	1292	159	Campania Isl., NNW of Rennision Isl.	52°56'30"N, 129°26'20"W
P9	1520	178	Odlum Isl., N of Starfish Isl., Q.C.S.	51°41'35"N, 128°07'05"W
P10	1583	185	N Pt., S of Gogit Point, Lyell Isl., Q.C.I.	52°39'55"N, 131°27'35"W
P11	1417	172	E Shore of Triquiet Isl., S of Edna & Spider Isl.	51°48'30"N, 128°14'W
P12	1474	173	Lancaster Reef, N. of Serpent Gr.	51°48'25"N, 128°10'30"W
P13	1369	171	SE Pts. of S Isl. off Edna Isl., S of Spider Isl., QCI.	51°49'20"N, 128°14'40"W
P14	1617	191	SW of Yakan Pt., SW of Tow Hill, McIntrye Bay	54°03'40"N, 131°51'00"W
P15	1630	192	S of Tlell st Pt., Hecate Strait, Q.C.I.	53°33'30"N, 131°55'40"W

Subspecies (OTU)	Coll. No.	Site	Location	Lat/Long
L1	380	66	2nd Westerly Isl., of Emily Islet Gr., Q.C.I.	51°01'35"N, 127°34'20"W
L2	1595	186	1st. Pt. NW of Vertical Pt., E of Small Islet, Q.C.I.	52°54'05"N, 131°37'40"W
L3	1602	189	Smith Pt., off Terrace of Q.C.I., N of Maple Isl.	53°15'10"N, 132°03'25"W
L4	1604	189	Smith Pt., off Terrace of Q.C.I. Museum,	53°15'10"N, 132°03'25"W
L5	1616	190	S end of Kumara Lake, S of Fife Pt., Rose Spit	54°05'25"N, 131°40'05"W
C1	710	COY8A	Coyhaique	45°15'S, 73°00'W[1]
C2	758	LCO5B	Lake Conquillo	38°45'S, 71°50'W[1]
C3	1114	2CAR3A	Lake General Carrera	46°28'S, 73°15'W[1]
C4	668	ANC2A	Ancud County	42°00'S, 73°50'W[1]
C5	739	COY21H	Coyhaique	45°15'S, 73°00'W[1]
C6	743	ILE2A	Island of Lemuy	42°30'S, 73°50'W[1]
C7	779	LON4F	Lonquimay	38°26'S, 71°14'W[1]
C8	781	MAUIA	Maullin	41°38'S, 73°37'W[1]
C9	797	TDC2B	Termas de Chillan	36°40'S, 72°10'W[1]
C10	814	TDT5H	Termas de Tolhuaca	38°15'S, 71°50'W[1]
C11	1064	2CPU1A	Carelmapu, 10 mi NNE of Ancud	42°10'S, 73°50'W[1]
C12	1068	2CAM1C	Camahueto River	42°55'S, 73°20'W[1]
C13	1072	2GBN1A	Poine Guabun	42°15'S, 74°05'W[1]
C14	1078	2CUC1A	Cucao County	42°42'S, 74°05'W[1]
C15	1113	2PUQ1A	Puquenun	42°08'S, 73°48'W[1]

[1] Latitude and longitude are estimated.

APPENDIX I: Original data matrix of presence (1) or absence (0) for 68 polymorphic band fragmentsd obtained from RAPD analysis generated using 12 primers with 54 plants including f. *pacifica* (P), f. *lucida* (L), ssp. *chiloenis* (C) and ssp. *sandwicensis from* Chapter 5.

1 68L 54L 0
743-870 743-840 743-720 743-700 743-680 743-330 743-320 743-290 743-260 743-250 743-240
743-230 746-720 748-780 748-740 756-1150 756-840 756-700 756-580 759-1500 743-920
748-720 759-1450 759-720 759-700 759-680 759-640 759-620 759-550 759-1500 759-690
772-1000 772-580 772-540 772-410 772-980 785-1000 785-980 785-560 785-390 785-350
785-400 790-620 790-560 790-520 790-480 790-350 790-600 790-500 790-320 790-240 792-740
792-700 792-680 792-400 792-370 792-420 795-720 795-680 796-450 798-600 798-540 798-370
798-340 798-310 798-300 756-650 796-680
CS1 CS2 CS3 CS4 CS5 CS6 CS7 CS8 CS9 CS10 CS11 CS12 CS13 CS14 CS15 CA1 CA2 CA3
CA4 CA5 SA1 SA2 SA3 SA4 SA5 SA6 SA7 SA8 SA9 SA10 SA11 SA12 SA13 SA14 SA15
HA1 HA2 HA3 HA4 HA5 HA6 HA7 HA8 HA9 HA10 HA11 HA12 HA13 HA14 HA15 HA16
HA17 HA18 HA19
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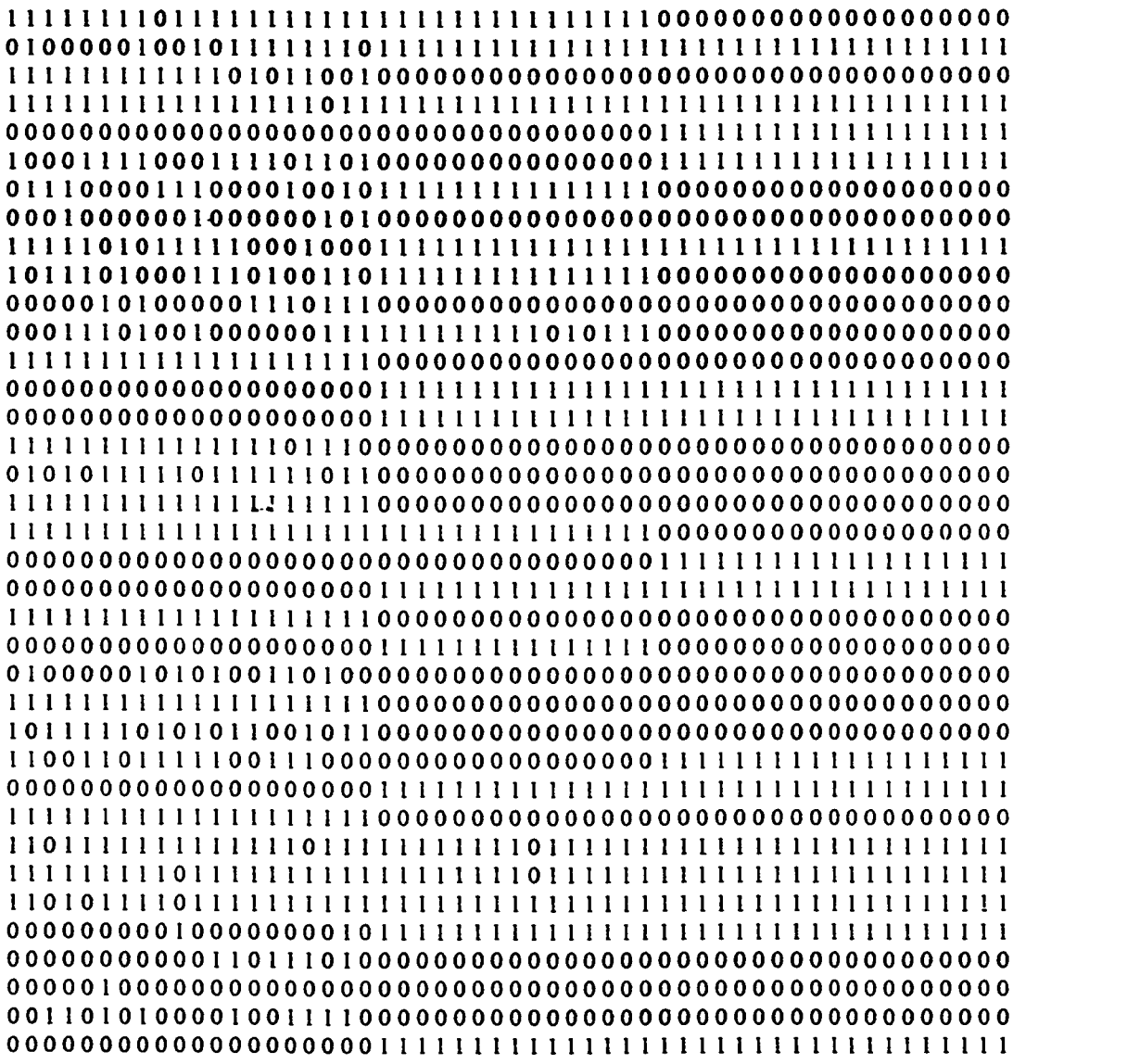


APPENDIX J: List of 54 OTU specimens including f. *pacifica* (P1-15), f. *lucida* (L1-5), ssp. *chiloensis* (C1-15) and ssp. *sandwicensis* (S1-19) with DAO accession numbers included in the molecular RAPD analysis.

OTU	DAO ACCESSION #
P1	708677
P2	708678
P3	708679
P4	708680
P5	708681
P6	708682
P7	708683
P8	708684
P9	708685
P10	708686
P11	708687
P12	708688
P13	708689
P14	708690
P15	708691
L1	708692
L2	708693
L3	708694
L4	708695
L5	708696
C1	708697
C2	708698
C3	708699
C4	708700
C5	707701
C6	707702
C7	707703
C8	707704
C9	707705
C10	707706
C11	707707
C12	707708
C13	707709
C14	707710
C15	707711
S1	707712
S2	707713
S3	707714
S4	707715
S5	707716
S6	707717
S7	707718
S8	707719
S9	707720
S10	707721
S11	707722
S12	707723
S13	707724
S14	707725
S15	707726
S16	707727
S17	707728
S18	707729
S19	707730

APPENDIX K:

Plate I. Photograph of strawberry plants grown in greenhouse at Agriculture and Agri-Food Canada, Ottawa.

Plate II. Photograph of *F. chiloensis* (a) ssp. *pacifica* with spreading hairs and (b) ssp. *lucida* with ascending hairs from North American

Plate III. Photograph of *F. chiloensis* (a) ssp. *sandwicensis* from Maui and (b) ssp. *chiloensis* shown in flower from South America.

Plate I. Photograph of strawberry plants grown in greenhouse at Agriculture and Agri-Food Canada, Ottawa.

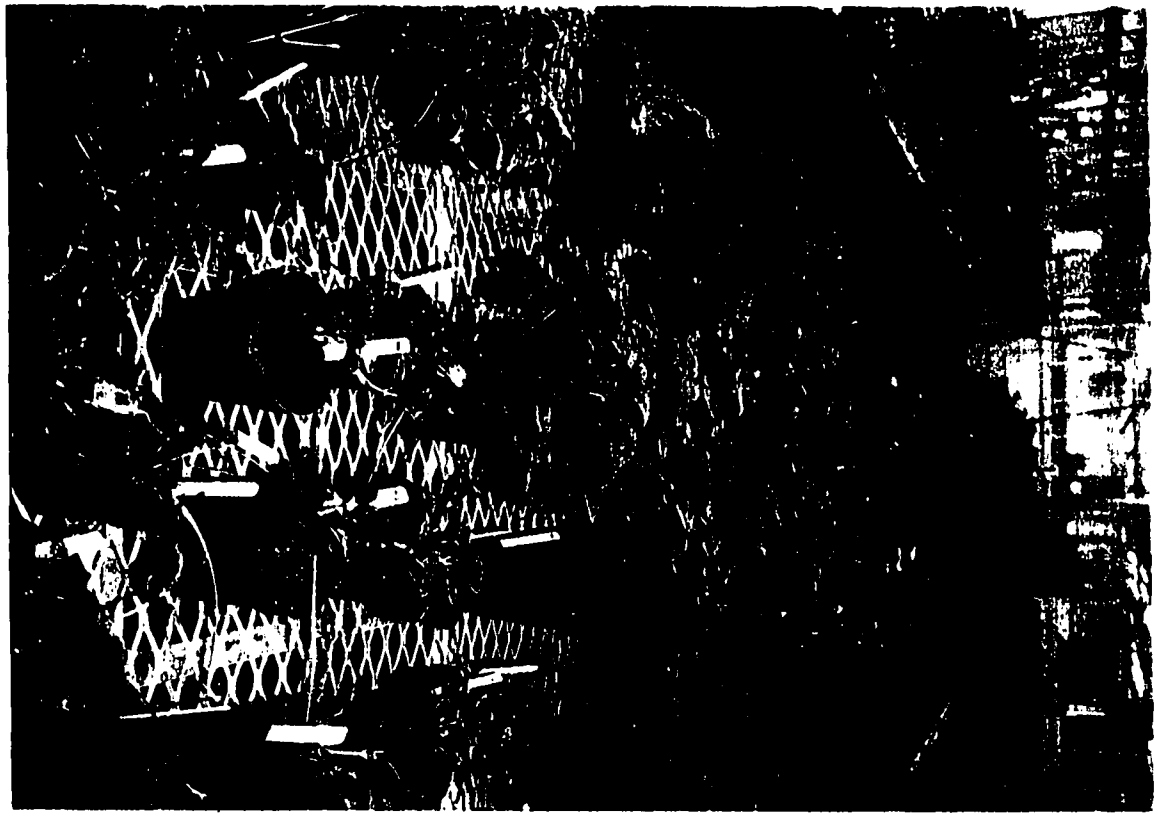
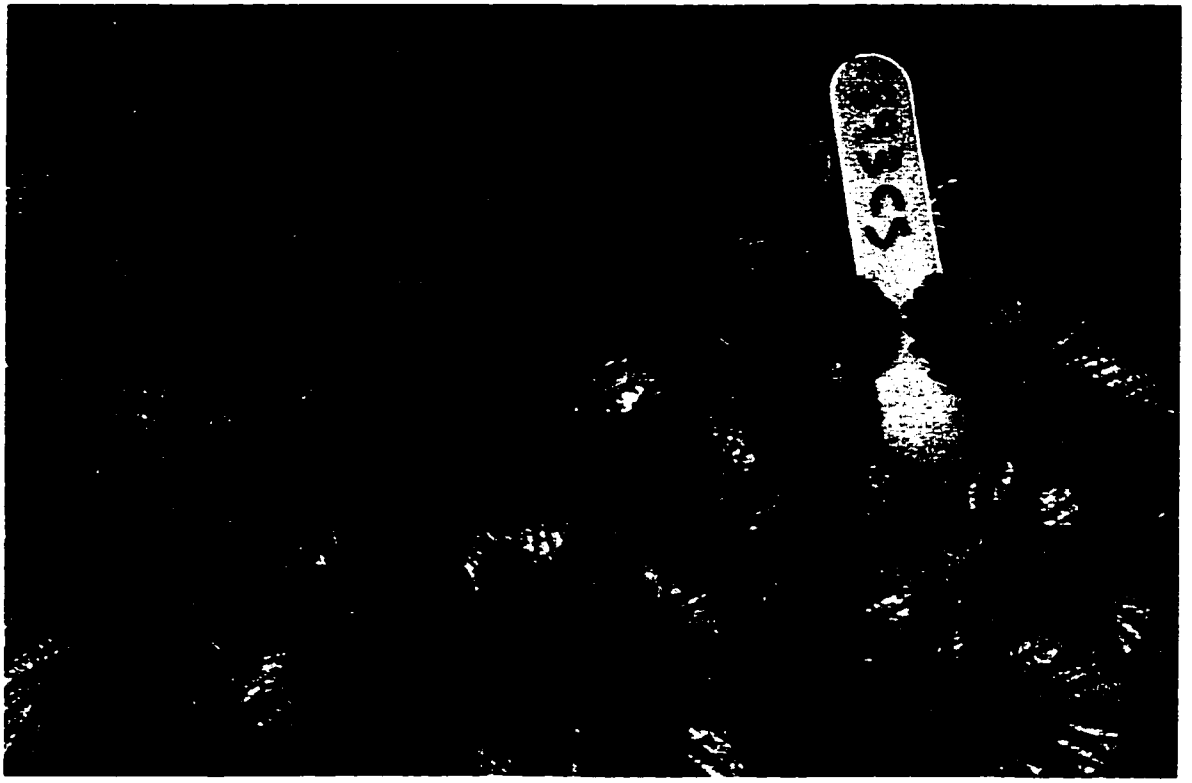


Plate II. Photograph of *F. chiloensis* (a) ssp. *pacifica* with spreading hairs and (b) ssp. *lucida* with ascending hairs from North American

(a)



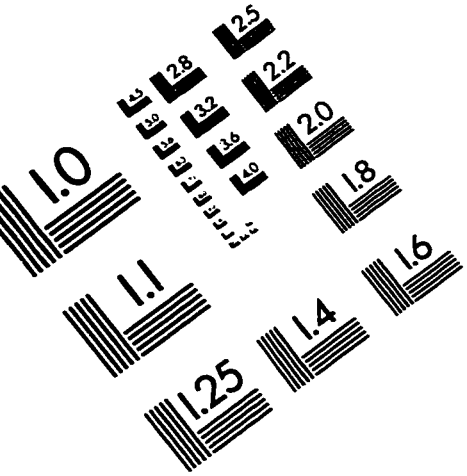
(b)

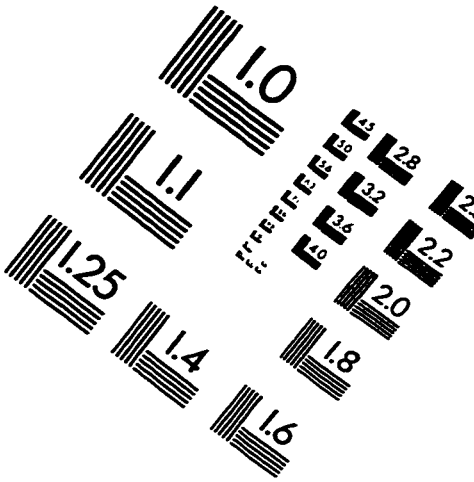
Plate III. Photograph of *F. chiloensis* (a) ssp. *sandwicensis* from Maui and (b) ssp. *chiloensis* shown in flower from South America.

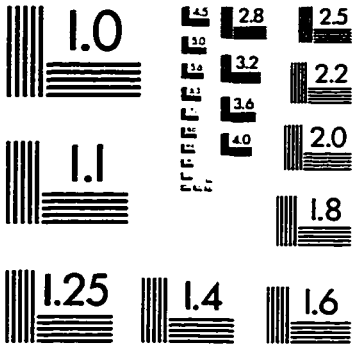
(a)

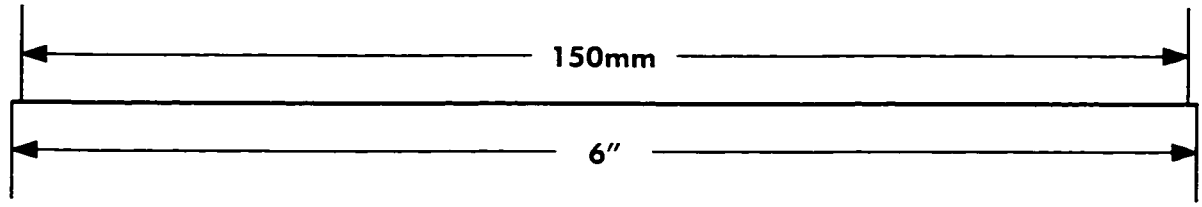
(b)

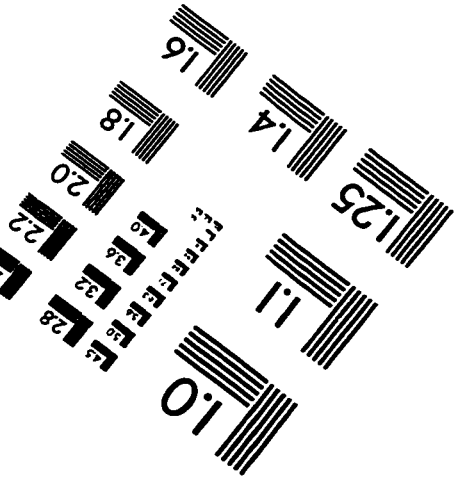
IMAGE EVALUATION
TEST TARGET (QA-3)

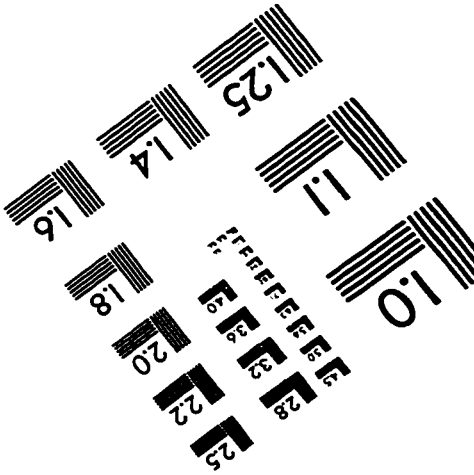












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