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GENETIC DIVERSITY AND EVOLUTIONARY RELATIONSHIPS WITHIN AND AMONG FIVE CYPRIPEDIUM (ORCHIDACEAE) SPECIES: INFERENCE FROM ALLOZYME ELECTROPHORESIS

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GENETIC DIVERSITY AND EVOLUTIONARY RELATIONSHIPS WITHIN AND AMONG FIVE *CYPRIPEDIUM* (ORCHIDACEAE) SPECIES: INFERENCE FROM ALLOZYME ELECTROPHORESIS

By

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ABSTRACT

GENETIC DIVERSITY AND EVOLUTIONARY RELATIONSHIPS WITHIN AND AMONG FIVE CYPRIPEDIUM (ORCHIDACEAE) SPECIES: INFERENCE FROM ALLOZYME ELECTROPHORESIS

By

Martha Ann Case

The Orchidaceae are one of the most morphologically diverse and species-rich families of flowering plants. Although tremendous morphological diversity exists within the family, very little is known about the extent of genetic variation within natural populations of orchids, or the degree of genetic differentiation among congeneric species. This study is one of the first examinations of allozyme variation in the Orchidaceae. It addresses current theories of orchid evolution concerning the levels of genetic diversity and taxonomic relationships within and among Cypripedium candidum, C. acaule, C. reginae, C. arietinum, and three North American varieties of C. calceolus.

Starch gel electrophoresis resolved the products of 14 isozyme loci which were used to quantify the degree of allozyme divergence within and among these Cypripedium taxa. Cypripedium calceolus and C. candidum have a high average genetic identity (Nei's genetic identity = 0.794) and appear to be a progenitor-derived species pair whereas all other species pairs have very low genetic identities ranging from 0.000 to 0.285. These low identity values are among the

lowest reported in the plant literature, and they indicate that the level of genetic divergence among Cypripedium species is substantially greater than most other congeneric plant species. Cypripedium arietinum, which is sometimes placed in a segregate genus Criosanthes, was more similar to C. calceolus and C. candidum than were C. reginae or C. acaule. This result indicates that C. arietinum should be retained in the genus Cypripedium. In contrast, varieties within C. calceolus show relatively little genetic divergence at isozyme loci. Intravarietal Nei's identity values ranged from 0.920 to 0.979, and they were not consistently higher than intervarietal values. The genetic data for these C. calceolus varieties are consistent with reports of genetic divergence among other sympatric infraspecific taxa and do not support current hypotheses that taxa within C. calceolus are distinct species.

The species also show considerable differences in Nei's diversity statistics. Average expected species-level heterozygosities were 0.244 (C. calceolus), 0.095 (C. acaule), 0.054 (C. candidum), 0.037 (C. reginae), and 0.000 (C. arietinum) with 19%, 16%, 7%, 35%, and 0% of the variation partitioned among populations, respectively. The relatively high level of heterozygosity in C. calceolus is also consistently high among each of its varieties. These results are discussed in the context of current theories on genetic variation in orchid populations and historical events which could have influenced the levels of variation.

Dedicated to the loving memory of my father

Richard James Case

1921 - 1987

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GENERAL INTRODUCTION

The results from an electrophoretic analysis of seven Cypripedium taxa are reported in two major subdivisions of this dissertation, Part 1 and Part 2. Each part is a complete paper with a separate abstract, introduction, materials and methods, results, discussion, and literature cited. Part 1 describes the extent of genetic divergence among C. calceolus, C. candidum, C. acaule, C. reginae, and C. arietinum, particularly with respect to the generic placement of C. arietinum. This paper also quantifies the amount of genetic variation found within Cypripedium populations and also analyzes the distribution of genetic variation among conspecific populations. These data are discussed in the context of: (1) current theories regarding the level of genetic variation in orchid populations, (2) comparable isozyme studies reported for other plant taxa, and (3) historical events that may have influenced the observed genetic patterns.

Part 2 focuses on the problem of the taxonomic status of Cypripedium calceolus var. pubescens, C. calceolus var. parviflorum, and C. calceolus var. planipetalum. It examines: (1) the degree of genetic divergence among these taxa, (2) the distribution of genetic variation among

populations within each taxon, and (3) the allele frequency differences among sympatric and allopatric populations of vars. parviflorum and pubescens. Several methods of analyses were employed including chi-square contingency analysis, unweighted pair-group method analysis, and principal components analysis. The results are discussed with respect to other allozyme studies of sympatric and allopatric infraspecific taxa reported in the plant literature.

PART 1

EXTENSIVE VARIATION IN THE LEVELS OF GENETIC DIVERSITY AND DEGREE OF RELATEDNESS AMONG FIVE SPECIES OF CYPRIPEDIUM

ABSTRACT

An electrophoretic analysis of 14 enzyme loci was conducted in order to describe the genetic variation contained within and among Cypripedium calceolus L., C. candidum Muhlenberg ex Willdenow, C. acaule Aiton, C. reginae Walter, and C. arietinum R. Brown in Aiton (Orchidaceae). While C. calceolus and C. candidum have a high average genetic identity (Nei's genetic identity = 0.794) and appear to be a progenitor-derived species pair, all other species pairs have very low genetic identities ranging from 0.000 to 0.285 which indicates extensive phylogenetic divergence. The species also show considerable differences in Nei's diversity statistics. Average expected species-level heterozygosities were 0.244 (C. calceolus), 0.095 (C. acaule), 0.054 (C. candidum), 0.037 (C. reginae), and 0.000 (C. arietinum) with 35% to 0% of the variation partitioned among populations. The low genetic variation and uniformity of populations present in several taxa could

have resulted from prior genetic bottlenecks which affected the ancestral populations. The results are discussed in the context of historical events that could have influenced the observed genetic patterns.

INTRODUCTION

The Orchidaceae are one of the most morphologically diverse and species-rich families of flowering plants (Dressler, 1981). Despite the family's tremendous diversity, very little is known about the level and maintenance of genetic diversity within natural populations, or the extent to which congeneric species differ at the allozyme level. This scarcity of electrophoretic data on the Orchidaceae may, to some extent, reflect the difficulties in working with orchids. These include their oftentimes sporadic occurrences (Case, 1987) in relatively small populations, and the difficulties in rearing many temperate orchids due to their long generation times (Curtis, 1943) and fastidious germination and nutrient requirements (Ballard, 1987; Stoutamire, 1990). Yet the unique combination of life-history and ecological characteristics which contribute to the difficulty in working on orchids also make them an especially interesting family to examine electrophoretically. For example, as a result of one successful pollination, some species of orchids can produce over a million dust-like, wind-dispersed seeds (Withner, 1988). This potential for long-distance dispersal in the family is thought to facilitate geographic isolation and, in combination with selection pressure to accommodate new pollinators, promote the extraordinary levels of speciation (Dressler, 1981). Isolated populations founded by one or a few individuals may also contain reduced

levels of genetic variation, especially if the population growth after the bottleneck remains slow (Nei, Maruyama, and Chakraborty, 1975). According to Gill (1989), the entire process leading to orchid population establishment may be viewed as a multiplicative series of improbable events. This has led to speculation that orchid populations should contain exceedingly low levels of genetic variation (Gill, 1989).

This study examines the genetic diversity within and among five taxa of the genus Cypripedium, a terrestrial genus containing 30 to 40 species of long-lived, herbaceous perennials (Luer, 1975). The genus Cypripedium, along with three other genera, comprise the subfamily Cypripedioideae, commonly known as the Slipper Orchids. This subfamily differs from the vast majority of orchids by having two fertile lateral anthers whereas the other major subfamilies contain one fertile median anther. This characteristic, along with other primitive characteristics in the subfamily, suggest that the lineage which has given rise to the Cypripedioideae diverged early in the evolution of the family (Correll, 1950; Dressler, 1981; Atwood, 1984). As typical members of the Cypripedioideae, the taxa in the present study have an inflated pouch-like labellum which insects enter through an opening in the top and become temporarily trapped inside. They pollinate the flower by first passing under the stigmatic region and then under one of two lateral anthers, each located near an exit hole in

the rear portion of the labellum. Self-pollination is generally thought to be improbable due to a physical separation of the reproductive structures (Luer, 1975). Furthermore, Nilsson (1979) observed that insect behavior which would lead to self-pollination in C. calceolus occurs infrequently. Therefore, it has been assumed that Cypripedium taxa routinely outcross, although geitonogamy may occur through the occasional production and cross pollination of two or three flowers on the same peduncle or from the cross-pollination of flowering shoots produced through clonal reproduction. In an examination of self-fertility in three Cypripedium taxa, Newhouse (1976) found that C. calceolus, C. reginae, and C. acaule produced fruit with apparently normal embryos after artificial self-pollination, and none of the taxa produced seeds after bagged flowers were emasculated.

Cypripedium taxa range from arctic areas to the subtropics of the Northern Hemisphere. Twelve species occur in North America, five of which are found predominantly or entirely within the northeastern United States and Canada (Luer, 1975). These five taxa are the subject of this paper and include Cypripedium calceolus L. (Yellow Lady's Slipper), C. candidum Muhlenberg ex Willdenow (White Lady's Slipper), C. arietinum R. Brown in Aiton (Ram's Head), C. acaule Aiton (Moccasin Flower), and C. reginae Walter (Showy Pink Lady's Slipper). In addition, results from a preliminary analysis of C. kentuckiense C. F. Reed are reported. This taxon is

known primarily from E. Kentucky to Louisiana and Arkansas (Atwood, 1985), and was only recently described by Reed (1981). Because relatively little is known about C. kentuckiense, the following paragraphs do not pertain to this species unless specifically mentioned. The initial electrophoretic results for this taxon are included in the discussion.

In North America, Cypripedium calceolus occupies the largest range of any Cypripedium taxon. Its range extends from Newfoundland to the Yukon and, in the eastern half of the United States, as far south as Louisiana. It also can be found in the greatest diversity of habitats including northern cedar-fir fens, limestone barrens, mixed deciduous forests, and roadside ditches. Similar to the ecological diversity, the morphological diversity in this taxon is likewise extensive and its relationship to species delimitation has been the subject of numerous papers and long-standing taxonomic debate (Sheviak, 1983). Due to this morphological diversity, three North American varieties have been recognized, vars. pubescens (Willdenow) Correll, parviflorum (Salisbury) Fernald, and planipetalum (Fernald) Victorin & Rousseau. Although populations of each of these varieties have been included in the C. calceolus populations analyzed in the present study, the genetic differences among these varieties will be the subject of a subsequent paper. Among the five taxa discussed in this paper, C. acaule has the second largest geographic range, which extends from

Newfoundland to the Great Bear Lake in the Northwest Territories and down to northeast Alabama. All other taxa occur predominantly east of North Dakota and occupy less total geographic area (Luer, 1975). Because of extensive geographic regions of sympatry and ecological requirements which can be similar, these taxa can occasionally be found in sympatric populations but only C. calceolus and C. candidum are known to produce natural hybrids. When this occurs, large hybrid swarms may develop which can be accompanied by extensive introgression (Actor, 1984). Cypripedium candidum and C. calceolus are also most similar morphologically whereas C. acaule, C. reginae, and C. arietinum are morphologically very distinct. They all have a chromosome number of 2n = 20 (Löve and Ritchie, 1966; Löve and Simon, 1968).

In a recent morphological analysis of evolutionary trends in the Cypripediodeae, C. acaule was considered to be divergent from C. candidum, C. calceolus, and C. reginae, and it was therefore placed in a different clade (Atwood, 1984). Cypripedium arietinum was also placed in a separate clade primarily because of three characteristics shared only by C. arietinum and C. plectrochilon Franch. (a very similar Asiatic taxon whose species distinction is questionable; Atwood, 1984). These characteristics are: (1) separate lateral sepals, (2) a spurred lip, and (3) a staminode which strongly resembles a fertile stamen. Based on a numerical analysis which incorporated these characters, Atwood (1984)

found C. arietinum (and therefore also C. plectrochilon) to be sufficiently dissimilar from other Cypripedium species that he proposed a transfer of these taxa to the segregate genus Criosanthes.

This paper examines the degree of allozyme divergence among C. arietinum and the four other Cypripedium species mentioned above. It also addresses the level of intraspecific variation in these taxa, how this variation is partitioned among populations, and how members of the Orchidaceae that have been examined electrophoretically compare to plants in other families with similar life history and ecological characteristics.

MATERIALS AND METHODS

Thirty one populations representing five taxa were analyzed by enzyme electrophoresis (Table 1.1). Since the Great Lakes region is particularly suitable for the growth of Cypripedium species (Case, 1987), most of the populations were collected from this region in a wide variety of habitats. For C. calceolus, however, collections were made from Newfoundland, Michigan, Indiana, Ohio, and Illinois in order to encompass a similar proportion of the total species range and morphological variation. Numbers of individuals sampled per population ranged from 12 to 88 and in the majority of cases, represented 25% to 100% of the estimated population size observed. Samples of fewer than 20 individuals represent a sample from every individual in the

TABLE 1.1. Location data, population collection numbers (PCN), and numbers of individuals sampled (n) for *Cypripedium* species analyzed.

PCN	n	Location
C. calceolus		
B01	20	Scioto Co., OH; 38°46'N 83°14'W, Shawnee State Forest
B03	20	Defiance Co., OH; T4N R5E S23, Defiance
B06	20	Vermillion Co., IL; T20N R12W S20, Danville
B07	20	Charlevoix C0., MI; T34N R7W S2, Bay Shore
B08	20	Lake Co., IN; T37N R9W, Hammond[a]
B09	20	Charlevoix Co., MI; T34N R7W S11, Bay Shore
B17	20	Baraga Co., MI; T50N R33W S21, Bovin
L01	18	Newfoundland, Canada; 19.3 km inland from Eddie's Cove along highway 430
L02	20	Newfoundland, Canada; 16.1 km inland from Eddie's Cove along highway 430
L03	20	Newfoundland, Canada; 3.2 km N of St. Barbe Bay along highway 430
L06	20	Newfoundland, Canada; along Green Garden Trail, seprentine area, Gros Morn National Park
V01	88	Shiawassee Co., MI; T5N R1E S20, Rose Lake Wildlife Research Area
V02	22	Presque Isle Co., MI; T33N R2E S18, Canada Creek
V03	47	Livingston Co., MI; T2N R5E[a]
V04	50	Livingston Co., MI; T2N R6E[a]

Table 1.1 (cont'd)

C. candidum		
C01	12	Kalamazoo Co., MI; T4S R11W S4, Hogset Lake
C02	20	Oakland Co., MI; T4N R8E S16, Davisburg
C03	20	Jackson Co., MI; T4S R3W S9, Brail Lake Creek
C04	15	Livingston Co., MI; T4N R4E S11 S12, Cohoctah
C05	40	Kalamazoo Co., MI; T1S R9W S30, Butterfield Lake
C. arietinum		
R01	20	Emmet Co., MI; T39 R4W S30, Cecil Bay
R02	29	Cheboygan Co., MI; T39N R3W S18 S19, Mackinaw City
R03	42	Presque Isle Co., MI; T34N R7E S14, Thompson's Harbor
R04	24	Leelanau Co., MI; Sleeping Bear Dunes National Lakeshore[a]
C. acaule		
A01	50	Emmet Co., MI; T35N R5W S25, Conway
A02	19	Chippewa Co., MI T51N R5W S32, Whitefish Point
A03	20	Washtenaw Co., MI; T2S R3E S4, Waterloo Recreation Area
A04	45	Presque Isle Co., MI; T33N R2E S18, Canada Creek

Table 1.1 (cont'd)

C. reginae		
S01	55	Clare Co., T17N R4W[a]
S02	19	Clinton Co., MI; T5N R1W S25, Rose Lake Wildlife Research Area
S03	23	Ingham Co., MI; T4N R1E S11, Coon Creek

[a]Exact locations witheld upon request of the proprietor

population.

Each population was first surveyed for approximate size and density of individuals and then tissue samples were collected from a representative proportion of flowering and non-flowering individuals. Care was taken to collect uniformly throughout the populations and also to avoid collecting from more than one ramet in a clump of leafy stalks suspected of being clonally reproduced. In addition to collecting plant material from naturally-occurring Cypripedium populations, leaf samples were also collected from three C. kentuckiense individuals cultivated by Frederick and Roberta Case in Saginaw, Michigan. Each plant had originated from a different state, Kentucky, Alabama, and Arkansas (F. Case, personal communication). For each sampled plant, one leaf tip (ca. 3 cm long) was removed and then divided into two equal portions which were each placed in a 1.5 ml centrifuge tube. All samples were kept on water ice in the field and then stored at -80 C in the lab until they were electrophoresed.

The leaf tissue was ground with a chilled mortar and pestle and an extraction buffer consisting of 0.1 M Tris-HCl, pH 7.5, 14 mM 2-mercaptoethanol, 1 mM EDTA (tetrasodium), 10 mM KCl, and 10mM $MgCl_2$ (Gottlieb, 1981a). In addition, 10 mg of polyvinylpyrrolidone-10 was added to the tissue at the time of grinding. The resulting slurry was absorbed onto filter paper wicks made from Whatman #17 paper (for buffer systems I and II described below) and

Whatman #3 paper (for buffer system III described below).

Ten enzyme systems were employed on 12% starch gels using three buffer systems. System I electrode buffer consisted of 0.029 M monohydrate lithium hydroxide with 0.192 M boric acid, pH 8.2, whereas the gel buffer consisted of one part electrode buffer and nine parts 0.051 M Tris with 0.007 M monohydrate citric acid, pH 8.4 (Crawford, 1982). This system resolved glutamate oxaloacetate transaminase (GOT), triose-phosphate isomerase (TPI), alcohol dehydrogenase (ADH), glutamate dehydrogenase (GDH), and superoxide dismutase (SOD). System II resolved phosphoglucomutase (PGM). The electrode buffer of this system consisted of 0.060 M sodium hydroxide with 0.300 M boric acid, pH 8.2, and the gel buffer consisted of 0.076 M Tris with 0.005 M monohydrate citric acid, pH 8.7 (Crawford, 1982). Malate dehydrogenase (MDH), phosphogluconate dehydrogenase (PGD), isocitrate dehydrogenase (IDH), and shikimate dehydrogenase (SKD) were resolved on a histidine system (system III) which consisted of 0.400 M citric acid trisodium salt, pH 7.0, for the electrode buffer, and 0.020 M histidine-HCl, pH 7.0, for the gel buffer (Gottlieb, 1981a). Electrophoresis was monitored by the position of a bromophenol blue marker band and was stopped when the dye had migrated 10.5 cm across the gel. Total time of electrophoresis varied from five to six hours for systems I and II, to eight to nine hours for system III. Agarose overlays, with minor modifications, followed the procedures of Soltis et al. (1983) and were

employed for all enzyme systems except GOT. This was a stain bath which followed the protocol of Crawford (1982). The preceding buffer and stain systems employed represent the optimal three out of approximately ten systems tested for enzyme separation, resolution and activity for the proteins assayed.

Genotypes were inferred directly from enzyme phenotypes by knowledge of the minimum number of isozymes present for given enzymes together with the knowledge of the quaternary structure of those enzymes (Gottlieb, 1981b, 1982; Weeden and Wendel, 1989). Enzymes which did not conform to the above expectations were dropped from the analyses. In cases where it was difficult to distinguish allozymes from isozymes, side by side comparison of leaf and pollen electromorphs were conducted following the recommendations of Weeden and Gottlieb (1979). Putative isozyme loci were given sequentially higher numbers for decreasing anodal migration of their protein products. Likewise, allele a was assigned to the most anodally migrating protein, b to the protein immediately following a, etc. Interpopulation verification of enzyme mobilities was conducted through side by side comparisons of allelic variants electrophoresed under identical conditions (i.e., on the same gel and with the same batch of extraction buffer).

Allele frequencies were calculated at the population levels and weighted according to sample size to produce species-level allele frequencies. From the populational

frequencies, Nei's (1978) genetic identity values were calculated for all pairwise population combinations using a modified version of GENESTAT (Whitkus, 1985). Identity values for each interspecific and intraspecific comparison were then averaged from the pairwise population values. The resulting average interspecific identity values were used to produce a UPGMA dendrogram following the procedure of Swofford and Olsen (1990).

For each population and species, the frequency of polymorphic loci (Pp and Ps, respectively), alleles per locus (Ap and As), and effective alleles per locus (Aep and Aes) were calculated using the procedure reported by Hamrick and Godt (1989). Within each species, the gene diversity statistics of Nei (1973), unbiased for sample size (Nei and Chesser, 1983), were calculated for all loci and averaged to give species estimates. This was calculated with the modified GENESTAT program cited above. The values reported here include total genetic diversity or species-level expected heterozygosity (Ht), average gene diversity within populations or average population-level expected heterozygosity (Hs), average gene diversity between populations (Dst), and the proportion of total genetic diversity attributable to among-population differentiation (Gst). Standard errors among loci were calculated for Ht, Hs, Dst, and Gst. For Gst, the jackknife method described in Weir (1990) was employed. To determine whether or not populations deviated significantly from Hardy-Weinberg

expectations, chi-square tests were performed for each polymorphic locus within each population. For loci with two alleles, Yates continuity correction was applied (Weir, 1990). When expectations for any genotypic class in any locus fell below one, the least common allele was collapsed into the adjacent allelic class until all genotypic classes had expectations greater than one. This procedure helped eliminate spurious inflation of the chi-square test statistic due to small numbers in the denominator (Hernández and Weir, 1989). For loci with greater than two alleles present, each heterozygote class was tested individually by comparing a squared estimated disequilibrium coefficient to an estimate of its variance (Hernández and Weir, 1989). Critical values for the desired alpha in this case were adjusted to account for correlated comparisons (Sokal and Rohlf, 1981). Significant ($p < 0.05$) and near significant ($0.05 < p < 0.10$) deviations are reported as well as the level of disequilibrium which is expressed by the fixation index, $F = 1 - (H/2pq)$, where H is the frequency of heterozygotes and p and q are the allele frequencies in the population (Hedrick, 1985). Gene flow estimates based upon the frequency of private alleles within populations were calculated by the method of Slatkin (1985) using a correction for sample size.

RESULTS

Ten enzyme systems clearly resolved the products of 14 putative loci used in this analysis. For all species analyzed, one isozyme was observed for ADH, SKD, GDH and SOD (except in C. reginae in which two regions of staining were observed for SOD). Two isozymes were observed for TPI, PGM, IDH, and PGD, however, PGD-1 and IDH-1 were not used due to low activity. Three isozymes were present for GOT. MDH had a clearly-defined slow locus (polymorphic in C. calceolus populations not included in this analysis) and several other clearly resolved but genetically equivocal faster electromorphs which were not used. Except for SOD and MDH, the number of isozymes and regions of staining were consistent across all taxa, but the resolution and activity of five enzymes varied greatly among species. Therefore, these loci had to be omitted from certain interspecific comparisons (see MDH-3, PGM-2, GOT-3, ADH, and GDH in Table 1.2).

Nei's average genetic identity values for intraspecific population comparisons ranged from 0.937 in C. calceolus to 1.000 in C. arietinum (Table 1.3). For interspecific comparisons, the highest average identity occurred for the C. calceolus-C. candidum comparison with an average identity of 0.794. This is in dramatic contrast to all other interspecific comparisons which have values of 0.000 for the C. arietinum and C. reginae pair to the second highest value of 0.285 for C. calceolus and C. arietinum. A visualization

TABLE 1.2. Species-level allele frequencies across 14 loci[a] and total individuals sampled per taxon (n).

	Cypripedium species				
Allele	CALC n = 425	CAND n = 107	ARIE n = 115	ACAU n = 134	REGI n = 97
TPI1a	-	0.028	-	-	-
b	0.344	0.967	-	-	-
c	-	-	-	-	1.000
d	-	-	1.000	0.974	-
e	0.656	-	-	0.026	-
f	-	0.005	-	-	-
TPI2a	-	-	-	0.063	-
b	-	-	-	0.015	-
c	-	-	1.000	-	-
d	-	-	-	-	1.000
e	0.748	1.000	-	0.918	-
f	0.252	-	-	-	-
g	-	-	-	0.004	-
SODa	-	-	-	1.000	-
b	-	-	1.000	-	-
c	-	-	-	-	1.000
d	1.000	1.000	-	-	-
MDH3	1.000	1.000	X	X	X
PGM1a	0.058	-	-	-	-
b	0.074	0.009	-	-	-
c	0.846	0.991	-	-	0.170
d	0.022	-	-	-	0.830
e	-	-	1.000	-	-
f	-	-	-	0.993	-
g	-	-	-	0.007	-
PGM2a	0.245	0.981	-	-	X
b	-	-	1.000	1.000	X
c	0.755	0.019	-	-	X
GOT1a	1.000	1.000	1.000	-	-
b	-	-	-	1.000	1.000

Table 1.2 (cont'd)

GOT2a	-	-	-	1.000	0.016
b	-	-	-	-	0.974
c	0.467	0.047	-	-	-
d	0.031	0.934	-	-	-
e	-	-	-	-	0.010
f	0.502	0.019	1.000	-	-
GOT3a	X	X	X	0.086	1.000
b	X	X	X	0.903	-
c	X	X	X	0.011	-
ADHa	0.048	0.079	X	-	X
b	-	-	X	1.000	X
c	0.948	0.921	X	-	X
d	0.001	-	X	-	X
e	0.001	-	X	-	X
f	0.002	-	X	-	X
IDHa	0.871	0.962	-	-	-
b	-	0.005	-	-	-
c	0.129	0.033	1.000	-	-
d	-	-	-	1.000	1.000
PGDa	0.002	0.023	-	-	-
b	0.861	0.963	1.000	-	-
c	-	-	-	-	1.000
d	0.002	-	-	-	-
e	0.135	-	-	0.004	-
f	-	0.014	-	-	-
g	-	-	-	0.716	-
h	-	-	-	0.280	-
SKDa	0.437	-	-	-	-
b	-	0.014	-	-	-
c	0.531	0.986	-	-	-
d	0.033	-	-	-	-
e	-	-	-	0.317	-
f	-	-	-	0.683	-
g	-	-	-	-	1.000
h	-	-	1.000	-	-
GDHa	X	X	1.000	-	-
b	X	X	-	1.000	-
c	X	X	-	-	1.000

[a]An X denotes unresolved loci.

TABLE 1.3. Average Nei's identity values and ranges for all intraspecific (along the diagonal) and interspecific (below the diagonal) comparisons among taxa, and the frequency of loci in which no alleles are shared (above the diagonal).

	CALC	**CAND**	**ARIE**	**ACAU**	**REGI**
CALC	0.937 (0.746-1.000)	0.000	0.600	0.727	0.889
CAND	0.794 (0.708-0.849)	0.995 (0.987-1.000)	0.600	0.909	0.889
ARIE	0.285 (0.235-0.359)	0.209 (0.191-0.231)	1.000 (1.000-1.000)	0.818	1.000
ACAU	0.079 (0.028-0.112)	0.091 (0.079-0.098)	0.208 (0.202-0.211)	0.975 (0.956-0.994)	0.636
REGI	0.027 (0.002-0.080)	0.027 (0.003-0.073)	0.000 (0.000-0.000)	0.247 (0.210-0.326)	0.978 (0.966-1.000)

of these values through UPGMA revealed that C. calceolus and C. candidum cluster first, followed by C. arietinum which joins the group at 0.247 (Figure 1.1). Cypripedium acaule and C. reginae form a separate cluster with an identity value of 0.247 and join the C. calceolus-C. candidum-C. arietinum group at an average identity of 0.072. The large genetic divergence among most taxa in the present study is further indicated by the high frequency of loci per taxon which contain moderate to high frequency unique alleles (mean Ua = 0.38 in Table 1.4), and by an average of 71% of the loci between species pairs which share no alleles (Table 1.3). This is in contrast to the high genetic identities between C. calceolus and C. candidum. In C. candidum, 79% of its alleles are also found in C. calceolus, and the remaining 21% (i.e., five alleles) are unique to C. candidum (Table 1.2).

In general, percent polymorphic loci, alleles per locus, and effective alleles per locus followed similar trends in population and species comparisons (Table 1.4). At the species level, 75% of the loci in C. calceolus were found to be polymorphic with 2.5 alleles per locus. Cypripedium candidum is next with values of 66.7% and 2.00, respectively, followed by C. acaule (46% and 1.77), C. reginae (18% and 1.27), and lastly, C. arietinum, which was monomorphic at all loci examined. Differences among the species were also found in the overall levels of genetic diversity (as measured by Ht) as well as how that diversity

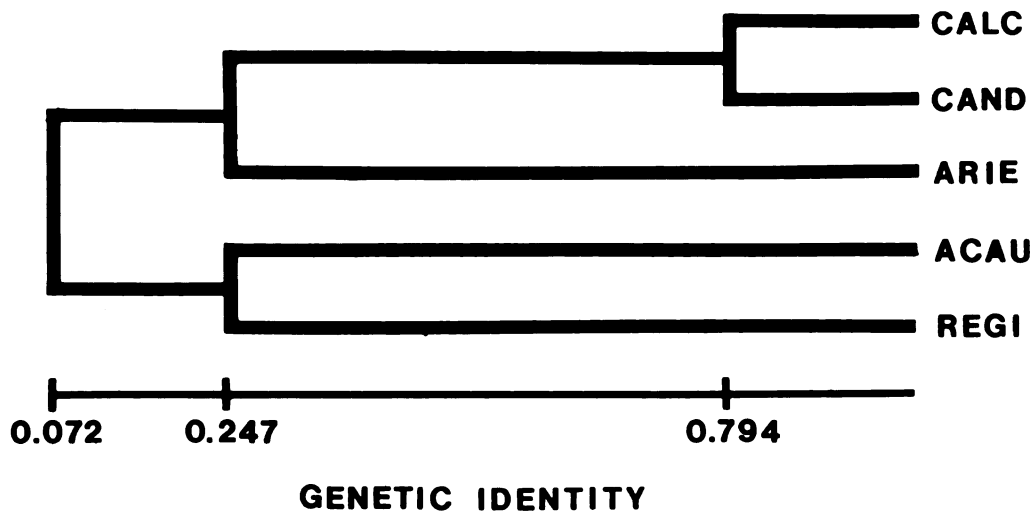

FIGURE 1.1. UPGMA dendrogram of Nei's (1978) genetic identities for C. calceolus (CALC), C. candidum (CAND), C. arietinum (ARIE), C. acaule (ACAU), and C. reginae (REGI).

TABLE 1.4. Frequency of polymorphic loci (P), alleles per locus (A), and effective alleles per locus (Ae) at the population (p) and species (s) levels, and the frequency of loci (Ua) which contain unique alleles with frequencies greater than 0.25 for each taxon.

Species	Pp (Ps)	Ap (As)	Aep (Aes)	Ua
C. calceolus	0.628 (0.750)	1.72 (2.50)	1.24 (1.33)	0.22
C. candidum	0.383 (0.667)	1.43 (2.00)	1.05 (1.06)	0.00
C. acaule	0.347 (0.462)	1.44 (1.77)	1.08 (1.12)	0.44
C. reginae	0.152 (0.182)	1.15 (1.27)	1.02 (1.05)	0.67
C. arietinum	0.000 (0.000)	1.00 (1.00)	1.00 (1.00)	0.56
Mean	**0.302 (0.412)**	**1.35 (1.71)**	**1.08 (1.11)**	**0.38**

is partitioned among populations. Cypripedium calceolus has an average Ht of 0.244 with 19% of this diversity distributed among populations (Table 1.5). Cypripedium acaule has the next largest level of diversity (Ht = 0.095) with 16% of its variation residing among populations. Cypripedium candidum and C. reginae have similar levels of diversity (Ht = 0.054 and 0.037, respectively) but may partition that diversity differently. Whereas only 7% of the total variation resides among populations in C. candidum, 35% of the total variation in C. reginae is distributed among populations. However, this relatively high Gst in C. reginae is due to a single polymorphic locus, PGM-1, which had an unusually high frequency of allele c in population S03.

Expected heterozygosity at the population level (i.e., Hs) followed the same species-level trend; C. calceolus populations were most heterozygous with an average Hs of 0.197 followed by C. acaule (0.080), C. candidum (0.050), and C. reginae (0.024; Table 1.5). Most loci were in good agreement with Hardy-Weinberg expectations, although significant deviations ($p < 0.05$, $p < 0.01$, or $p < 0.001$) occurred in eight cases distributed across five loci and six populations (Table 1.6). Five of the cases involve heterozygote excess. A deficiency of homozygotes was also the most common form of deviation from Hardy-Weinberg expectations in the nearly significant cases ($0.05 < p < 0.10$). Estimates of Nm (the average number of migrants

TABLE 1.5. Gene diversity statistics for polymorphic Cypripedium species; Ht is total diversity, Hs and Dst are within- and among-population diversity, respectively, and Gst is the proportion of variation attributable to among-population differentiation; standard errors of loci are in parentheses.

Species	**Ht**	**Hs**	**Dst**	**Gst**
C. calceolus	0.244 (0.054)	0.197 (0.045)	0.047 (0.012)	0.194 (0.025)
C. candidum	0.054 (0.020)	0.050 (0.017)	0.004 (0.002)	0.069 (0.032)
C. acaule	0.095 (0.046)	0.080 (0.037)	0.015 (0.011)	0.164 (0.075)
C. reginae	0.037 (0.032)	0.024 (0.020)	0.013 (0.013)	0.349 (0.316)

TABLE 1.6. Significant ($p < 0.05$, 0.01 or 0.001) and near significant ($0.05 < p < 0.10$) single-locus deviations from Hardy-Weinberg equilibria and the level of negative or positive disequilibria expressed by the fixation index.

	Locus						
Population	**TPI-1**	**PGM-1**	**PGM-2**	**GOT-2**	**PGD**	**IDH**	**SKD**
B01			0.394[a]				
B03	-0.539[b]						-0.504[a]
V03	-0.382[b]	-0.5337[d]	0.319[b]			-0.324[a]	
L06			0.560[b]				
C01				1.000[c]			
S03		-0.468[a]					
A02					0.513[a]		
A04					-0.312[a]		
A01							-0.363[b]
A03							-0.539[b]

[a] = $p < 0.10$; [b] = $p < 0.05$; [c] = $p < 0.01$; [d] = $p < 0.001$

exchanged between local populations per generation) for intraspecific populations were 3.62 (C. reginae), 4.07 (C. acaule), 8.36 (C. calceolus) and 11.07 (C. candidum).

DISCUSSION

Interspecific relationships--The electrophoretic data reveal an extremely wide range in the degree of relatedness among the five taxa examined. While Nei's intraspecific average identities ranged from 0.937 in C. calceolus to 1.000 in C. arietinum, the average interspecific genetic identity values ranged from 0.000 for the C. reginae-C. arietinum comparison to 0.794 for the C. calceolus-C. candidum comparison. Hence the difference in magnitude between average intraspecific and interspecific values indicate clearly demarcated and genetically distinct taxa. This is further indicated by an average congeneric identity value of 0.197 which is among the lowest yet reported in the plant literature. This value is considerably lower than 0.670, the average congeneric identity reported for plant species (Gottlieb, 1977, 1981b; Crawford, 1983).

Too few studies have examined electrophoretic variability in the Orchidaceae to enable generalizations, but it is interesting to note that other studies on the family also have reported unusually low genetic identities. For example, 11 species in the genus Orchis from Italy displayed interspecific identity coefficients in the range of 0.000 to 0.767 with a mean congeneric identity of 0.194 (Scacchi, De

Angelis, and Lanzara, 1990). Epipactis is another orchidaceous genus which has relatively low identity values (Scacchi, Lanzara, and De Angelis, 1987). These authors found that congeneric identity among three Italian species was 0.470; two of these species pairs had low interspecific identities (0.289 and 0.336), whereas the third pair had a higher genetic identity of 0.784. In non-orchidaceous studies which have examined genetic identities among several congeneric species, low genetic identities among species pairs similar to that found in Cypripedium occasionally occur but are usually confined to a small percentage of the species comparisons or involve a relatively small number of species (e.g., Manos and Fairbrothers, 1987; Olmstead, 1990; but see also Nickrent, 1986). In part, this may reflect a tendency for investigators to examine species which are morphologically difficult to delineate because of recent phylogenetic separation. Recently, however, intercontinental disjunct species pairs which may represent relic populations from ancient more widespread distributions have been examined. Values reported for these species pairs are oftentimes lower than the 0.67 mean reported for congeneric species, but are still higher than many of the Cypripedium or Orchis species pairs (Figure 1.2). For example, Vogelmann and Gastony (1987) report congeneric identity values among disjunct Agastache species ranging from 0.51 to 0.75. Still, other reports of vicariad species pairs are lower, such as 0.305 for a Liquidambar species

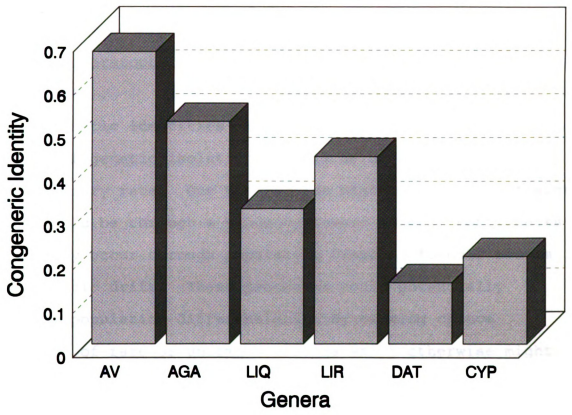

FIGURE 1.2. Average congeneric Nei's identity values for intercontinental species pairs within the genera Agastache (AGA), Liquidambar (LIQ), Liriodendron (LIR), and Datisca (DAT) compared to the average congeneric identity values reported for plant species in general (AV) and for Cypripedium species in this study (CYP).

pair (Hoey and Parks, 1991), and 0.434 for a Liriodendron species pair (Parks and Wendel, 1990). Two of the lowest non-orchid congeneric identity values yet reported are 0.142, among two intercontinental disjunct Datisca species (Liston, Rieseberg, and Elias, 1989), and 0.087 among North American Ceratophyllum demersum L. and C. echinatum A. Gray (Les, 1989).

Low genetic identities may reflect a relatively long period of genetic isolation as well as an accelerated evolutionary rate. One way orchids might evolve at a faster rate would be through a tendency toward genetic bottlenecks which may occur through population crashes, founder events and genetic drift. These processes could potentially promote population differentiation by causing chance fixation of rare or uncommon alleles which otherwise might remain in low frequency. Although these processes may have played an important role in the evolution of Cypripedium taxa at some point in time, several lines of evidence suggest that at least a long period of genetic isolation has contributed substantially to those species pairs with low genetic identities. First (with the exception of the C. candidum-C. calceolus pair), an average of 71% of the loci among pairwise species comparisons share no alleles. This, together with a high proportion of moderate to high frequency unique alleles in most taxa (Table 1.4), suggest that they have existed long enough in genetic isolation to evolve very dissimilar genomes. This is a very different

pattern of genetic identities than is often found among species that have undergone recent adaptive radiation. In island endemics, for example, extensive morphological and ecological diversity is often accompanied by little divergence at isozyme loci (Helenurm and Ganders, 1985).

The occurrence of closely related taxa in the Old World is another observation which suggests that most Cypripedium taxa analyzed have experienced a long period of phylogenetic separation. Cypripedium calceolus is represented in Europe and Asia by C. calceolus var. calceolus whereas C. reginae and C. arietinum are morphologically and ecologically so similar to the East Asian C. flavum Hunt & Summerh. and C. plectrochilon Franch., respectively, that species distinctions have not yet been resolved (Sing-Chi, 1983). It has been suggested that these species pairs have descended from a common stock which once had a continuous range (Sing-Chi, 1983). Cypripedium acaule also shows similar morphological affinities with a group of Old World species (Atwood, 1984) whereas in North America it bears no close resemblance to any extant species. The distribution of Cypripedium in the Northern Hemisphere together with the low genetic identities found is consistent with the hypothesis that the Cypripediodeae is an old lineage, and further suggests that the genus Cypripedium may also be very old.

Although most taxa have evolved extensive genetic differentiation, the allozyme data do not suggest that C.

arietinum represents the most divergent taxon as the morphology would indicate. On the contrary, C. arietinum and C. calceolus have the second highest interspecific identity value of all interspecific comparisons (Table 1.3). Cypripedium acaule and C. reginae have approximately the same degree of genetic divergence from each other as does C. arietinum and C. calceolus (or C. candidum), but they have an extremely low identity with the C. calceolus-C. candidum-C. arietinum group (Figure 1.1). Therefore, a transfer of C. arietinum into a segregate genus based on genetic identity values would also necessitate the creation of two additional segregate genera for C. reginae and C. acaule. Since relatively little electrophoretic analysis has been conducted on closely related genera, little is known about the degree of genetic divergence genera commonly have. Likewise, until a larger body of electrophoretic literature is available for the Orchidaceae from which to compare interspecific values, the Cypripedium taxa in the present study are best interpreted by the allozyme data as highly divergent but congeneric.

Intraspecific diversity--Intraspecific measures of genetic diversity also vary greatly among the taxa examined, again reaching above and below the averages reported for comparable flowering plants (Figure 1.3). Cypripedium calceolus had the highest levels of genetic variation for all of the genetic parameters examined. At the species level, 75% of its loci were polymorphic with an average of

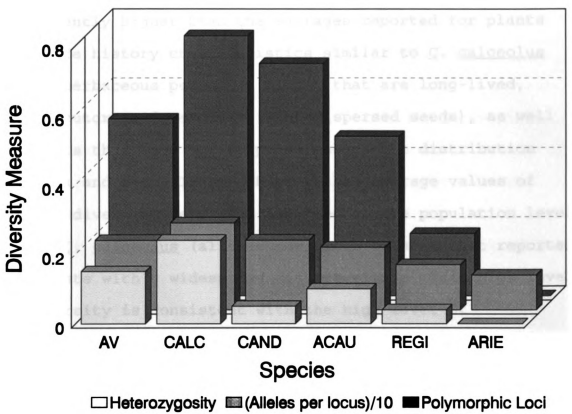

FIGURE 1.3. The frequency of expected heterozygotes, alleles per locus, and the frequency of polymorphic loci at the species level for C. calceolus (CALC), C. candidum (CAND), C. acaule (ACAU), C. reginae (REGI), and C. arietinum (ARIE) compared to the average species-level diversity measures reported for plants in general (AV).

2.5 alleles per locus, and its species-level heterozygosity (Ht) was 24.4%. These values are considerably higher than the averages reported for other plant taxa (50.5%, 1.96, 14.9%, respectively; Hamrick and Godt, 1989). They are also consistently higher than the averages reported for plants with life history characteristics similar to C. calceolus (i.e., herbaceous perennial plants that are long-lived, animal-outcrossed, and have wind-dispersed seeds), as well as plants that have a widespread geographic distribution (Hamrick and Godt, 1989). Higher than average values of genetic diversity were also observed at the population level within C. calceolus (alleles per locus equaled that reported for plants with a widespread distribution). This high level of diversity is consistent with the high level of morphological and ecological diversity found within this taxon. Moreover, each variety of C. calceolus has generally higher than average levels of genetic diversity as measured by percent polymorphic loci, alleles per locus and heterozygosity estimates (M. Case, Part 2). Therefore, the high level of variation within C. calceolus does not result from the inclusion of divergent infraspecific taxa, rather it is due to the high level of variation within each variety. The distribution of genetic variation within this species is also consistent with the observation that this taxon is predominantly outcrossed. In an outcrossed species, the majority of species-level variation, as measured by expected heterozygosity, often resides within as

opposed to among populations (Hamrick and Godt, 1989). Similarly, only 19% of the variation in C. calceolus resides among populations, which is equal to the average value reported for animal-outcrossed taxa (Hamrick and Godt, 1989). Moreover, the results of single-locus tests for deviations from Hardy-Weinberg expectations (Table 1.6) support the idea that self-pollination does not frequently occur within this taxon. These tests indicate that there were few significant deviations from the expected levels of heterozygosity across the vast majority of single-locus tests, and that when significant deviations did occur, they were most often in the direction of excess heterozygosity. In summary, the genetic diversity within C. calceolus is partitioned in a manner that is typical of a widespread, outcrossed, long-lived herbaceous plant, but it contains higher levels of diversity than average values reported for species with similar characteristics.

In contrast to the high levels of variability in C. calceolus, the remaining taxa have either reduced levels of polymorphic loci or very low levels of heterozygosity. While C. candidum has the second highest percentage of polymorphic loci at both the species and population levels (66.7%, 38.3%, respectively), the levels of heterozygosity at these loci are greatly reduced to 5.4% at the species level and 5.0% at the population level. Although low heterozygosities are typical for species with narrow distributions (Hamrick and Godt, 1989) similar to C.

candidum, the average values reported for such species are still twice as large as those reported for C. candidum. Since the majority of differences in levels of heterozygosity in plants has been found to be due to the proportion of polymorphic loci rather than differences at individual loci (Hamrick and Godt, 1989), C. candidum is somewhat atypical in this regard. In this species, low heterozygosity values do not result from a low proportion of polymorphic loci, but rather from very low heterozygosities at a large number of polymorphic loci. This discrepancy may result from past introgression of alleles from C. calceolus to C. candidum, which may have increased the number of polymorphic loci in C. candidum but perhaps not enough to significantly affect overall levels of heterozygosity. More extensive introgressive hybridization of these two taxa may occur in Brandon, Manitoba (Actor, 1984). In an allozyme survey of this population, Actor found very high levels of heterozygosity in C. candidum (34%), C. calceolus (37%) and in individuals that were morphologically classified as hybrids (37%). Therefore, there is nearly a seven-fold increase in heterozygosity in the Brandon population of C. candidum compared to Michigan populations when heterozygosities are recalculated with the five loci that were common to both studies. Either there are much higher levels of introgression in the Brandon population, or strong regional differences occur for the level of heterozygosity in C. candidum.

The relatively high genetic identity of C. calceolus and C. candidum (0.794) and the reduced level of variation present in all Michigan populations of C. candidum as compared to C. calceolus may reflect a progenitor-derived species relationship. In a progenitor-derived pair of relatively recent origin, the derived taxon frequently exhibits a subset of the variation present in the progenitor as well as unique alleles which commonly exist in low frequency (Gottlieb, 1977; Pleasants and Wendel, 1989). This expectation is consistent with the variation found in C. candidum. Percent polymorphic loci, alleles per locus, and heterozygosity at the species level are reduced in C. candidum compared to C. calceolus by 11%, 20% and 78%, respectively. Moreover, 79% of the alleles in C. candidum also occur in C. calceolus, whereas the remaining 21% are unique to C. candidum and exist in low species-level frequencies (0.005-0.028). If a C. calceolus population gave rise to C. candidum, then the alleles present in highest frequency in the progenitor population would also have the highest probability of being present in the derived taxon (e.g., see Stephanomeria in Gottlieb, 1973). Consistent with this expectation, the highest-frequency alleles present in C. calceolus are to a large extent present as the highest-frequency or second highest-frequency alleles in C. candidum. For example, six of the nine mutually polymorphic loci share the same highest frequency alleles, and the highest frequency alleles at two loci in C.

candidum are the second highest frequency alleles in C. calceolus. Only one locus in C. candidum has a high frequency allele that is present at low frequency in C. calceolus.

Although only a very limited amount of material was available for analysis, another progenitor-derived species pair may occur. In the three C. kentuckiense individuals examined, no polymorphism occurred across the 12 loci examined and all individuals were fixed for the same alleles. These alleles were, at every locus, the highest frequency alleles present in Cypripedium calceolus var. pubescens, the North American taxon it most closely resembles and with which it is sympatric (see Atwood, 1985, who recognizes C. calceolus var. pubescens as a distinct species). One of the most notable features of C. kentuckiense is its relatively large size, which is rivaled only by a Chinese species, C. wilsonii Rolfe. It has been suggested that the latter is a potential close relative of C. kentuckiense (Atwood, 1985). Although a larger genetic sample of C. kentuckiense is needed before any conclusions can be drawn, it is doubtful that C. wilsonii is more closely related to C. kentuckiense than is C. calceolus.

Many other potential progenitor-derived species pairs have been identified through enzyme electrophoresis (see reviews by Gottlieb, 1981b; Crawford, 1983; Pleasants and Wendel, 1989) including the orchid pair, Epipactis microphylla and E. helleborine (Scacchi, Lanzara, and De Angelis 1987).

Epipactis microphylla was found to exhibit no variability within or between populations and, with the exception of one unique allele, had the most common alleles of E. helleborine. Founder events were implicated in the depauperate nature of the E. microphylla populations. If rapid speciation is common in the Orchidaceae as proposed by Dressler (1981), then many such progenitor-derived species pairs should occur. Therefore, protein electrophoresis has great potential for testing this idea in genera like Stanhopea whose species apparently undergo rapid speciation due to strong selection for different pollinators (Dodson, 1962). Cypripedium acaule maintains the second highest levels of species and population heterozygosities (0.095 and 0.080, respectively) which represent a 60% decrease from values observed for C. calceolus. Similarly, percent polymorphic loci at the species and populational levels are reduced by 38% and 45%, respectively in C. acaule compared to C. calceolus. However, in comparison with other plant taxa which have a regional to widespread distribution and similar life history characteristics, these values are close to the averages reported for polymorphic loci, but are consistently smaller than the averages reported for heterozygosity levels (Hamrick and Godt, 1989). Gill (1989) has predicted that C. acaule populations in particular should contain extremely low levels of genetic variation because the combined probability that a given seed will reach a new site, survive the numerous highly vulnerable

stages to adulthood, and then be self-pollinated to establish a population is thought to be extraordinarily small. Although heterozygosities in C. acaule are lower than the averages reported for comparable species, this taxon is not genetically depauperate. Furthermore, all Cypripedium taxa in this study have comparable life history characteristics but the levels and distribution of genetic variation among them differ dramatically. This suggests that factors other than their life-history characteristics, at least in part, also affect the variation.

The remaining taxa, C. reginae and C. arietinum, have remarkably low levels of genetic variation and could be viewed as genetically depauperate. Cypripedium arietinum contained no polymorphic loci whereas only two of 11 loci were polymorphic in C. reginae. Although overall species-level heterozygosity in C. reginae was 0.037, which is only slightly smaller than the levels in C. candidum, the distribution of the variation in the two taxa is very different. Whereas C. candidum has 7% variation of its variation distributed among populations, C. reginae has on average 35% of the variation distributed this way. However, the two polymorphic loci in C. reginae give very different estimates (Gst for PGM-1 = 45%; Gst for GOT-2 = 1%) because a PGM-1 allele which is rare in two populations is common in population S03. When this population is omitted from the analysis, average Ht and Hs each lower to approximately 0.012 and virtually no variation is spread among

populations. The two polymorphic loci also give nearly identical estimates for Ht, Hs, and Gst when population S03 is removed.

The overall striking features of these five Cypripedium species are the common occurrence of low genetic identities, low intraspecific variation (with the exception of C. calceolus), and an average of 10% (omitting population S03) of the total species-level variation in polymorphic taxa distributed among populations. Likewise, only 7% of the total species-level variation (Scacchi, De Angelis, and Lanzara, 1990) in Orchis populations and 3.3% in Epipactis helleborine (Scacchi, Lanzara, and De Angelis, 1987) are distributed in such a manner. In species which are likely to have isolated populations founded by one or a few individuals, genetic drift should act to increase the variance in allele frequencies among populations (Nei, 1987). This would cause a high proportion of the total species-level variation to be distributed among populations rather than within. This population structure has been observed in Lisianthius skinneri (Hemsl.) Kuntze, a tropical, perennial shrub found in moist, mid-elevational forests from Guatemala to Panama (Sytsma and Schaal, 1985). Lisianthius skinneri is very similar to Cypripedium taxa in that populations are likely to be founded by few individuals, and they are markedly patchy in distribution and small in size. Consistent with the genetic expectations of this population structure, no genetic variation was found

in Lisianthius skinneri at the populational level and different populations were fixed for different allelic variants across the polymorphic loci. Therefore, 100% of the total species-level variation was found to reside among populations. Unlike L. skinneri, Cypripedium species have average to below average Gst values for perennial, outcrossed taxa with wind-dispersed seeds, and they are only one third as large as the average Gst value reported for plants which routinely undergo inbreeding (e.g., see Gst value reported by Hamrick and Godt, 1989, for self-pollinating species). Therefore, genetic drift or other evolutionary processes promoting differentiation among populations do not seem to have contributed more to among-population variation in Cypripedium taxa than in other comparable plant taxa. This is a surprising result given the apparent isolated nature of many orchid populations, and further suggests that these orchid taxa, despite the propensity for small, widely spaced populations, have or have had average to relatively high levels of interpopulational gene flow.

In these Cypripedium taxa, Slatkin's (1985) measure of gene flow is also suggestive of high levels of gene flow. This method uses the average frequency of alleles which are confined to one population (i.e., "private alleles") for an estimation of the average number of migrants exchanged between local populations [(Nm)est]. In this analysis, the Cypripedium taxa have values ranging from 3.62 in C. reginae

to 11.07 in C. candidum with an average (Nm)est of 6.8. This is very similar to other averages reported for plants which have either winged seeds [(Nm)est = 4.313] or wind-pollination mechanisms [(Nm)est = 5.380], and is considerably higher than average values reported for plants which have animal-outcrossed pollination mechanisms [(Nm)est = 0.801; Hamrick, 1987].

The general uniformity of populations and the frequent low levels of heterozygosity in these Cypripedium taxa may have several contributing factors. In C. candidum, it is likely that the low levels of heterozygosity and among-population variation are, to some degree, related to the possible origin of the taxon. However, the lack of any close relatives of C. arietinum, C. acaule, and C. reginae on this continent and their likely occurrence in Asia, in addition to the abundance of moderate to high frequency species-specific unique alleles, suggest that these taxa are phylogenetically very old. Therefore, it is unlikely that the lowered diversity in these taxa is due to their recent derivation.

Another factor which could potentially influence the genetic variability may be the degree to which adaptation to different ecological niches has maintained or reduced variation within each taxon. One hypothesis regarding this states that adaptation to different aspects of the environment is responsible for the maintenance of species variation (Van Valen, 1965). Therefore, species which can

exploit a variety of niches should, in theory, contain larger amounts of variation than should species with more restricted requirements. This has been termed the "niche width-variation hypothesis" (Babbel and Selander, 1974). In a broad sense, a correlation of niche width and genetic diversity can be seen with Cypripedium. The species able to exploit the widest array of habitats (i.e., C. calceolus) is the most genetically diverse, while the most ecologically restricted species (i.e., C. candidum) is among the lowest in species diversity as measured by expected heterozygosity. Lastly, historical factors may also have had substantial affects on the observed genetic variation within these taxa. Since the majority of each species' range now occurs north of the last Wisconsin continental ice sheet border (Luer, 1975; Péwé, 1983), their current northward distributions represent range expansions that have occurred within the last 11,000 years (Webb, Cushing, and Wright, 1983). For some species, glaciation may have substantially reduced their geographic range and restricted them to small, isolated populations. Under these conditions, there would be loss of genetic variation within populations due to genetic drift. Moreover, populations which now occur in glaciated areas could have been derived from a single genetically depauperate progenitor population. In the extant populations, this would cause reduced genetic variation within populations and produce a high level of uniformity among populations. Species which could inhabit a

wider array of habitats or persist in a wider geographical area during glaciation may not have been as affected by glacial processes on a species level. In these species, genetic variation could be lost in certain geographical areas but the persistence of numerous populations across a wide geographic range could, in theory, preserve species-level variation (Templeton et al., 1990).

Among the North American taxa, Cypripedium calceolus extends farthest south of the glacial boundary, occurring in most eastern states south to Louisiana. If this relatively large southern range and ecological diversity reflects an equally extensive range during the late Quaternary, then more species-level variation may have been maintained in C. calceolus than in the remaining taxa which are less ecologically diverse and may also have had relatively less available habitat during glaciation. The extreme of this is seen in C. arietinum, a less ecologically diverse species which has a current range totally within the glacial boundary. The corresponding complete lack of genetic variation in this taxon at the species level is suggestive of a genetic bottleneck which has eliminated variation in a population ancestral to the current populations.

The low heterozygosities together with the uniformity of C. reginae (excluding consideration of population S03) and C. candidum populations are also suggestive of genetic bottlenecks which occurred in ancestral populations. In the latter species, only 7% of its variation is partitioned

among populations, whereas its close relative, C. calceolus, maintains 19% of the variation in this way. Although Hamrick and Godt (1989) found that the breeding system and the method of seed dispersal accounted for the largest proportion of variation in Gst values, these two variables are not likely to differ appreciably among C. candidum and C. calceolus and thus account for the difference. However, if the current populations of C. candidum were derived from a common ancestral population, then the allele frequencies may reflect those of the ancestral population. A recent, common evolutionary history of populations could also lead to inaccurate estimates of gene flow as measured by the frequency of private alleles. When genetically isolated populations are founded from a common ancestral population, and have not yet reached a genetic and demographic equilibrium, then the frequencies of rare alleles in those populations are determined by the time since radiation rather than by current levels of gene flow (Slatkin, 1985). This would lead to an overestimate of the actual gene flow.

It is also likely that patterns of gene flow among present populations are different than those patterns which occurred when populations were recolonizing newly deglaciated territory. Since these species are infrequently found in mature forest but are found most abundantly in more open areas of transient successional stages (Case, 1987), it is likely that open areas after glacial retreat may have been particularly favorable to the establishment of these orchid

taxa. Their potential for long-distance dispersal would also have aided their fast recolonization. The current populations which are now confined to a patchy mosaic of favorable habitats may therefore represent "relic" populations from a much more contiguous range in a former landscape that was more conducive to gene flow.

Glacial disturbances and other Pleistocene phenomena have apparently reduced the genetic variation in many taxa such as Pinus resinosa Aiton (Fowler and Morris, 1977; Simon, Bergeron, and Gagnon, 1986), Acacia mangium Willd. (Moran, Muona, and Bell, 1989), Salicornia europea L. (Wolff and Jefferies, 1987), Trillium nivale Riddell (Bayer et al., 1987), Cirsium pitcheri (Torr.) Torr. & A. Gray (Loveless and Hamrick, 1988), and Sullivantia species (Soltis, 1982). In Sullivantia, three of the four taxa analyzed contained no variation and the remaining taxon contained extremely low levels. These populations currently are restricted to unglaciated regions near Pleistocene glacial margins and colonized these sites after the Wisconsin glaciation. It was suggested that founder events occurred during colonization. This, along with their narrow endemic nature, may have contributed to the decreased levels of variation in those populations (Soltis, 1982).

The levels of variation in Cypripedium taxa are complex, ranging broadly across values reported for plant taxa. Despite life histories that are similar, consideration of them alone can be misleading in predicting levels of

variation contained within and among populations. Likewise, evolutionary inference from current measurements of gene flow and population structure may not be accurate because current conditions may not be the same as those under which the populations were originally established. In Cypripedium it is apparent that each taxon has a unique evolutionary history and that inference of genetic variation based upon common life-history characteristics is not generalizable to all taxa.

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PART 2

HIGH LEVELS OF ALLOZYME VARIATION WITHIN *CYPRIPEDIUM CALCEOLUS* AND LOW LEVELS OF DIVERGENCE AMONG ITS VARIETIES

ABSTRACT

An electrophoretic analysis of 12 isozyme loci was completed to determine the level of genetic variation within and degree of genetic divergence among populations of *Cypripedium calceolus* var. *pubescens*, *C. calceolus* var. *parviflorum*, and *C. calceolus* var. *planipetalum*. In addition, the genetic relationship between mixed and pure populations of vars. *pubescens* and *parviflorum* was determined. Differences were found among pure populations of these taxa in the level of heterozygosity and distribution of genetic variation. Variety-level heterozygosity over all loci ranged from 0.180 in var. *pubescens* to 0.274 in var. *parviflorum*, whereas the proportion of variation distributed among populations ranged from 5.3% in var. *planipetalum* to 17% in var. *parviflorum*. Only slight levels of genetic divergence were found among these taxa, which resulted from allele frequency differences among the varieties rather than the presence of ubiquitous

unique alleles. Unweighted pair-group method analysis of Nei's genetic identities and a principal components analysis of allele frequencies both revealed a tendency of the populations to cluster with their respective varieties. However, var. parviflorum displayed a large variance in population differentiation. Intravarietal Nei's identity values, which ranged from 0.920 to 0.979, were not consistently higher than intervarietal values (0.900 - 0.954). These results are comparable to other reports of genetic divergence among sympatric infraspecific taxa and suggest that vars. parviflorum, pubescens, and planipetalum be considered members of a single species.

INTRODUCTION

Cypripedium calceolus L. is a diploid, outcrossed member of the Cypripedioideae (Orchidaceae) with a vast geographic range. In the Old World, it extends throughout much of Europe and Asia, occurring as far north as northern Sweden and as far south as south central China (Hultén and Fries 1986). In North America, the major portion of its range extends from Newfoundland across Canada (including portions of the northernmost United States), down to Louisiana (in the eastern portion of the US), and west to New Mexico in the southernmost states (Luer 1975). Over this extensive geographic range, C. calceolus displays extreme morphological and ecological variability which has led to the recognition of a myriad of poorly-defined taxa (see review by Correll 1938). According to Sheviak (1983), this species has "generated probably more thought and contradictory pages of print than any other North American orchid." One aspect of this controversy concerns the delimitation of two out of the three currently recognized North American taxa. These taxa have been viewed by different authors as: (1) varieties of C. calceolus [C. c. var. parviflorum (Salisb.) Fern. and C. c. var. pubescens (Willd.) Correll; e.g., Luer 1975], (2) two distinct species (C. parviflorum Salisb. and C. pubescens Willd.; e.g., Atwood 1985), and (3) varieties of one species which is separate from the European C. calceolus [C. parviflorum Salisb. var. parviflorum and C. p. var. pubescens (Willd.)

Knight; e.g., Sheviak 1992]. For purposes of this paper, they will be referred to as C. calceolus var. parviflorum and C. calceolus var. pubescens.

This controversy, however, concerns more than the problem of rank. It also includes the long-standing debate over which characters can reliably be assigned to each entity (see review by Newhouse 1976). This problem is further complicated by regional variation in characters as well as variation in the presence of isolating mechanisms. For example, in some regions introgression or the presence of plants with intermediate morphologies can be found. This apparently occurs between varieties of C. calceolus (Harms 1986; Sheviak 1992; Stoutamire 1967) as well as between C. calceolus and other closely-related species (Actor 1984; Klier et al. 1991; Sheviak 1992). In other geographic regions, morphologically distinct varieties occur in sympatry without the presence of plants with intermediate morphologies (Atwood 1985; Newhouse 1976). It is the latter situation that has led some researchers to view these varieties as distinct species (e.g., Atwood 1985).

As commonly interpreted (e.g., Case 1987), var. pubescens is the most widely distributed taxon and is found throughout the range reported above for North American C. calceolus. It can occur in a variety of habitats (e.g., from swamp to upland), and can be highly variable in height (10 - 80 cm). The labellum is often large (i.e., more than 4 cm long) and the lateral petals and sepals are greenish yellow, and

spotted or streaked with maroon. In contrast, var. parviflorum tends to be smaller in overall size with a labellum generally less than 4 cm long. It has deep mahogany-maroon petals and sepals, and is most often found in wet habitats. Other characters less commonly used to distinguish the varieties are reviewed in Newhouse (1976). Since the definition of var. parviflorum has been in debate, its geographic range is also uncertain. Some authors feel that it is largely restricted to the northeastern United States (e.g., Luer 1975) while others maintain that its range extends from the eastern United States throughout Canada to Alaska (e.g., Harms 1986). Sheviak (1992), Atwood (1985), and Case (1987) have discussed regional variation in var. parviflorum and suggested the possibility that additional taxa may be included within it.

Cypripedium calceolus var. planipetalum (Fern.) Vict. & Rousseau is a third commonly recognized taxon which is also of questionable rank. This taxon is shorter than the other taxa (up to 20 cm tall) and is characterized by having shorter, broader, and relatively untwisted lateral petals. It was originally described from the limestone barrens of Newfoundland, but plants with similar form have been found to occur sporadically outside Newfoundland (Atwood 1985; Luer 1975; Sheviak 1983) from as far away as British Columbia (Correll 1950). Atwood (1985) recognizes this taxon as a variety whereas Sheviak (1992) considers it to be an extreme expression of var. pubescens which does not

warrant varietal status.

In this paper the results of an electrophoretic analysis of vars. pubescens, parviflorum, and planipetalum from the northeastern United States and southeastern Canada are reported. This analysis was conducted to determine whether vars. parviflorum, pubescens, and planipetalum show levels of genetic differentiation that are most commonly associated with congeneric species or conspecific taxa. Furthermore, this study addresses the following additional genetic questions: (1) Are there differences among these varieties in their overall levels of genetic variation or the proportion of genetic variation distributed among populations? (2) How genetically similar are populations which contain pure morphological forms of vars. pubescens and parviflorum to populations that are intermediate in morphology to vars. pubescens and parviflorum or contain potential hybrids of these varieties? (3) Do the allele frequencies found within some sympatric populations of vars. parviflorum and pubescens indicate that these varieties maintain distinct gene pools in sympatry?

MATERIALS AND METHODS

Allozyme electrophoresis was carried out on 554 individuals from 22 populations. The samples included seven populations of var. pubescens (populations A through G, Table 2.1), four populations each of var. parviflorum (H through K), and var. planipetalum (L through O), and seven

TABLE 2.1. Location information, number of individuals sampled, and reference letter for populations of C. calceolus var. pubescens (PUB), C. calceolus var. parviflorum (PARV), C. calceolus var. planipetalum (PLAN), and populations containing putative hybrids or more than one morphological entity. Populations with two taxa listed, followed by a B, indicate populations with both morphological taxa and intermediate morphologies present. Populations with two taxa listed, followed by an H, indicate the presence of individuals with intermediate morphologies only.

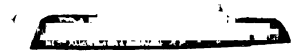
Morphological Taxon	Population Reference Letter	Individuals Sampled	Location
PUB	A	20	Charlevoix Co., MI; T34N R7W S2
---	B	20	----------; T34N R7W S11
---	C	20	Lake Co., IN; T37N R9W[a]
---	D	20	Baraga Co., MI; T50N R33W S21
---	E	20	Scioto Co., OH; 38°46'N 83°14'W
---	F	20	Vermillion Co., IL; T20N R12W S20
---	G	20	Defiance Co., OH; T4N R5E S23
PARV	H	88	Shiawassee Co., MI; T5N R1E S20
---	I	22	Presque Isle Co., MI; T33N R2E S18
---	J	47	Livingston Co., MI; T2N R5E[a]

Table 2.1 (cont'd)

---	K	50	----------; T2N R6E[a]
PLAN	L	18	Newfoundland, Canada; 19.3 km inland from Eddies Cove
---	M	20	----------; 16.1 km inland from Eddies Cove
---	N	20	----------; Green Garden trail head, Gros Morne
---	O	20	----------; 3.2 km N of St. Barbe Bay
PARV	P[b]	20	Chippewa, Co., MI; T41N R7E[a]
PUB	Q[b]	20	----------
PARV/PUB-B	R	20	----------; T42N R6E S10
PARV/PUB-H	S	19	Presque Isle Co., MI; T35N R5E S28
PARV/PUB-B	T	10	Ontario, Canada; Manitoulin Island S end of Manitowaning Bay
PARV/PUB-B	U	20	Ingham Co, MI; T4N R1E[a]
PARV/PUB-H	W	20	Cheboygan Co., MI; T35N R2W S27

[a] Exact locations witheld upon request of proprietor

[b] Populations P and Q are sympatric populations; no individuals with morphologies intermediate to vars. pubescens and parviflorum were observed at this site



"mixed" populations which could potentially consist of introgressed or hybridized individuals. Determinations of vars. pubescens and parviflorum followed the character descriptions of Case (1987) whereas delimitation of var. planipetalum followed the concept of Luer (1975). Populations chosen to represent each variety were from collection sites where all the individuals present in the population could unambiguously be identified to the same variety. Moreover, these sites were chosen in locations where additional varieties of C. calceolus were not commonly found. These populations are referred to as "pure" populations. If any population could not easily be determined to variety or if more than one variety was present, the population was either excluded from the study or classified in one of the mixed categories. Mixed populations were categorized on the basis of the morphological forms and the degree of intermediacy present at the site. Three different categories of mixed populations were observed: (1) populations composed of individuals with "pure" and intermediate morphologies (populations R, T, and U; Table 2.1), (2) populations with individuals having intermediate morphologies only (S and W), and (3) sympatric populations with only pure forms of each variety present and no intermediate morphologies (P and Q). Voucher specimens were collected for the vast majority of populations and are held at MSC.

The number of individuals sampled per population ranged

from 10 to 88. Larger sample sizes were obtained from larger populations whereas sample sizes under 20 correspond to smaller populations in which a sample was obtained from every individual observed. Sampling occurred uniformly throughout the populations and included collections from flowering and non-flowering individuals in pure populations. Since plants with only juvenile or adult vegetative morphologies could not be determined to variety, samples were taken only from flowering individuals in the mixed populations. No more than one sample was taken from a cluster of individuals suspected of being clonally reproduced. For each plant sampled, one leaf tip (approximately 3 cm long) was removed, divided in half, and placed in two 1.5 ml centrifuge tubes. The samples were kept on water ice in the field and subsequently frozen at -80 C in the laboratory until electrophoresis.

Leaf tissue was ground with a Tris-HCl extraction buffer following the protocol of Gottlieb (1981a). Three buffer systems were used in combination with nine staining systems. Glutamate oxaloacetate transaminase (GOT), triose-phosphate isomerase (TPI), alcohol dehydrogenase (ADH), and superoxide dismutase (SOD) were resolved on a lithium-borate system (Crawford 1982), whereas malate dehydrogenase (MDH), phosphogluconate dehydrogenase (PGD), isocitrate dehydrogenase (IDH) and shikimate dehydrogenase (SKD) were resolved on a histidine system (Gottlieb 1981a). A sodium-borate system (Crawford 1982) was used for

phosphoglucomutase (PGM). The staining protocols for all but GOT followed the agarose overlay procedures of Soltis et al. (1983). A stain bath was used for GOT which followed the procedure of Crawford (1982). Other details regarding the electrophoretic procedure of C. calceolus have been described elsewhere (M. Case, Part 1).

Genotypes were inferred directly from enzyme phenotypes by knowledge of the minimum number of isozymes present for given enzymes together with a knowledge of the quaternary structure of those enzymes (Gottlieb 1981b, 1982; Weeden and Wendel 1989). Side-by-side comparison of leaf and pollen electromorphs enabled a distinction of allozymes from isozymes (Weeden and Gottlieb 1979). Enzymes which did not conform to the above expectations were dropped from the analyses. In cases where two isozymes were present, the fastest anodally migrating isozyme was given the designation 1, and each successively slower isozyme was given a sequentially higher number. Likewise, the allele corresponding to the fastest migrating allozyme was designated a, the second fastest was designated b, etc. Interpopulation verification of enzyme mobilities was conducted through side-by-side comparisons of allelic variants electrophoresed on the same gel with the same batch of extraction buffer.

Four measures of diversity were calculated: percent polymorphic loci (P), alleles per locus (A), and the diversity statistics of Nei (1973) and Wright (1984).

Percent polymorphic loci was calculated for each population (Pp), variety (Pv), and species as a whole (Ps) by dividing the number of loci which had at least two alleles present by the number of loci analyzed. Likewise, alleles per locus (A) was calculated for each population (Ap), variety (Av), and the species as a whole (As) by dividing the total number of alleles present by the total number of loci analyzed. Nei's (1973) diversity statistics, unbiased for sample size (Nei 1978), were calculated over all loci with GENESTAT (Whitkus 1985) for each variety and the species. Reported here are Ht, the total genetic diversity or expected heterozygosity, and Gst, the proportion of the total genetic diversity attributable to among-population differentiation. The standard error for Gst was calculated by the jackknife method described in Weir (1990). In addition, a hierarchical analysis of population differentiation (Wright 1984) was employed to determine the proportion of variation distributed within populations, among populations within varieties, and among varieties. These values were calculated with BIOSYS-1 (Swofford and Selander 1989), but expressed in the form of Nei (1973) where each diversity component represents a proportion of the total diversity.

Nei's unbiased genetic identity (Nei 1978), Rogers distance (Rogers 1972), and Cavalli-Sforza and Edwards arc distance (Cavalli-Sforza and Edwards 1967) were calculated for all populations (pure with mixed) with BIOSYS-1. Several clustering techniques were calculated by the above

program and applied to the identity and distance measures. These included unweighted pair-group method analysis (UPGMA), weighted pair-group method analysis (WPGMA), single linkage, and complete linkage. Nei's genetic identity (I) with UPGMA resulted in the lowest percent standard deviation (Fitch and Margoliash 1967) and highest cophenetic correlation coefficient (Sokal and Rohlf 1962) and are reported herein.

In addition to UPGMA, a principal component analysis calculated by NTSYS (Rohlf 1988) was applied to the pure populations to evaluate the existence of potential groupings. For this analysis, population allele frequencies were standardized for populations (unit variance, zero mean) and a variance-covariance matrix was produced from the standardized allele frequencies. The first three principal component axes were extracted and populations A through O were plotted.

Contingency table analysis was completed for each locus with BIOSYS-1 to determine the extent of allele frequency heterogeneity among varieties and among populations within varieties. For intervarietal comparisons the analysis was conducted on pooled population allele frequencies and on the allele frequencies of the sympatric populations P and Q. All populations were tested for deviations from Hardy-Weinberg equilibria by calculating exact probabilities with BIOSYS-1. This procedure uses population allele frequency estimates to calculate the probability that a

given genotypic array at a single locus will occur under the assumption of random mating. Lastly, the possibility of a correlation between Nei's (1978) genetic distance and geographic distance was tested for all intravarietal populations using Spearman's Rank correlation (Sokal and Rohlf 1981).

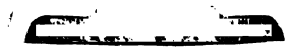
RESULTS

Nine staining systems resolved the products of 17 putative loci, 12 of which were used in this analysis. Three isozymes were found for GOT and MDH although GOT-3 was not used due to poor resolution and MDH-1 and MDH-2 were not used because of genetically equivocal isozyme patterns. Two isozymes were found for TPI, PGM, IDH, and PGD but low activity in PGD-1 and IDH-1 resulted in the omission of these isozymes from the analysis. One isozyme was found for SOD, SKD, and ADH. All individuals were scored for each of the 12 reliable isozymes. Only two loci, GOT-1 and SOD, were monomorphic in all populations examined. MDH-3 was polymorphic (with a rare allele) only in populations consisting of more than one variety.

At the species level, Cypripedium calceolus had 75% of its loci polymorphic with an average of 63% of the loci polymorphic in populations (Table 2.2). Similar values were found in each of the three varieties (i.e., 75% of the loci were polymorphic in each variety, and population-level polymorphism ranged from 60% in var. pubescens to 69% in

TABLE 2.2. Diversity measures for C. calceolus. Percent polymorphic loci (P) and alleles per locus (A) are given for the varietal (v) and species (s) levels, and the average population level (p). Nei's diversity statistics were calculated over all loci for vars. pubescens (PUB), parviflorum (PARV), planipetalum (PLAN), and for the species as a whole (All). Presented here are total varietal- or species-level expected heterozygosity (Ht), and the proportion of the total variation attributable to among population variation (Gst). Jackknife standard errors (S.E.) for Nei's diversity statistics are in parentheses.

Taxon	**Pp (Pv)**	**Ap (Av)**	**Ht (S.E.)**	**Gst (S.E.)**
PUB	0.60 (0.75)	1.63 (2.25)	0.180 (0.045)	0.084 (0.015)
PARV	0.69 (0.75)	1.79 (2.33)	0.274 (0.062)	0.171 (0.044)
PLAN	0.63 (0.75)	1.79 (2.25)	0.233 (0.062)	0.053 (0.073)
	Pp (Ps)	**Ap (As)**		
All	0.63 (0.75)	1.72 (2.50)	0.244 (0.054)	0.194 (0.025)



var. parviflorum). An average of 2.5 alleles per locus was observed at the species level (Table 2.2). Among the varieties, var. parviflorum had the highest number of alleles per locus (Av = 2.33), followed by vars. pubescens and planipetalum (Av = 2.25 for each). One unique allele was found in var. pubescens, two were found in var. parviflorum, and one was found in var. planipetalum (Table 2.3). The average frequencies of these rare unique alleles were 0.004, 0.004, and 0.006 in each of the taxa, respectively. Furthermore, a large percentage of high-frequency alleles was shared among the varieties. The percentage of shared alleles ranged from 69% among vars. parviflorum and var. planipetalum to 81% among vars. parviflorum and var. pubescens, with the average allelic frequency of unshared alleles ranging from 0.003 to 0.086 (Table 2.3).

The highest varietal-level expected heterozygosity was found in var. parviflorum (0.274) followed by var. planipetalum (0.233), and var. pubescens (0.180; Table 2.2). The majority of this variation resides within populations for each variety. Variety planipetalum is least structured with only 5.3% of its variation distributed among populations, and var. pubescens has a similar value of 8.4%. Variety parviflorum, however, has a two-fold increase in structure with 17% of its variation distributed among populations (Table 2.2). The hierarchical analysis of C. calceolus revealed that 81% of the total species-level

TABLE 2.3. Status of shared alleles among varieties and unique alleles within varieties of Cypripedium calceolus.

Taxon or Comparison	Percent Shared Alleles	Average Allelic Frequency of Unshared Alleles	Number of Unique Alleles	Average Frequency of Unique Alleles
PUB	-	-	1	0.004
PARV	-	-	2	0.004
PLAN	-	-	1	0.006
PARV/PUB	81	0.003	-	-
PARV/PLAN	69	0.056	-	-
PUB/PLAN	71	0.086	-	-

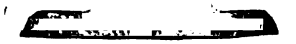
variation is found within populations, 12% of the variation is within varieties, and only 7% is distributed among varieties.

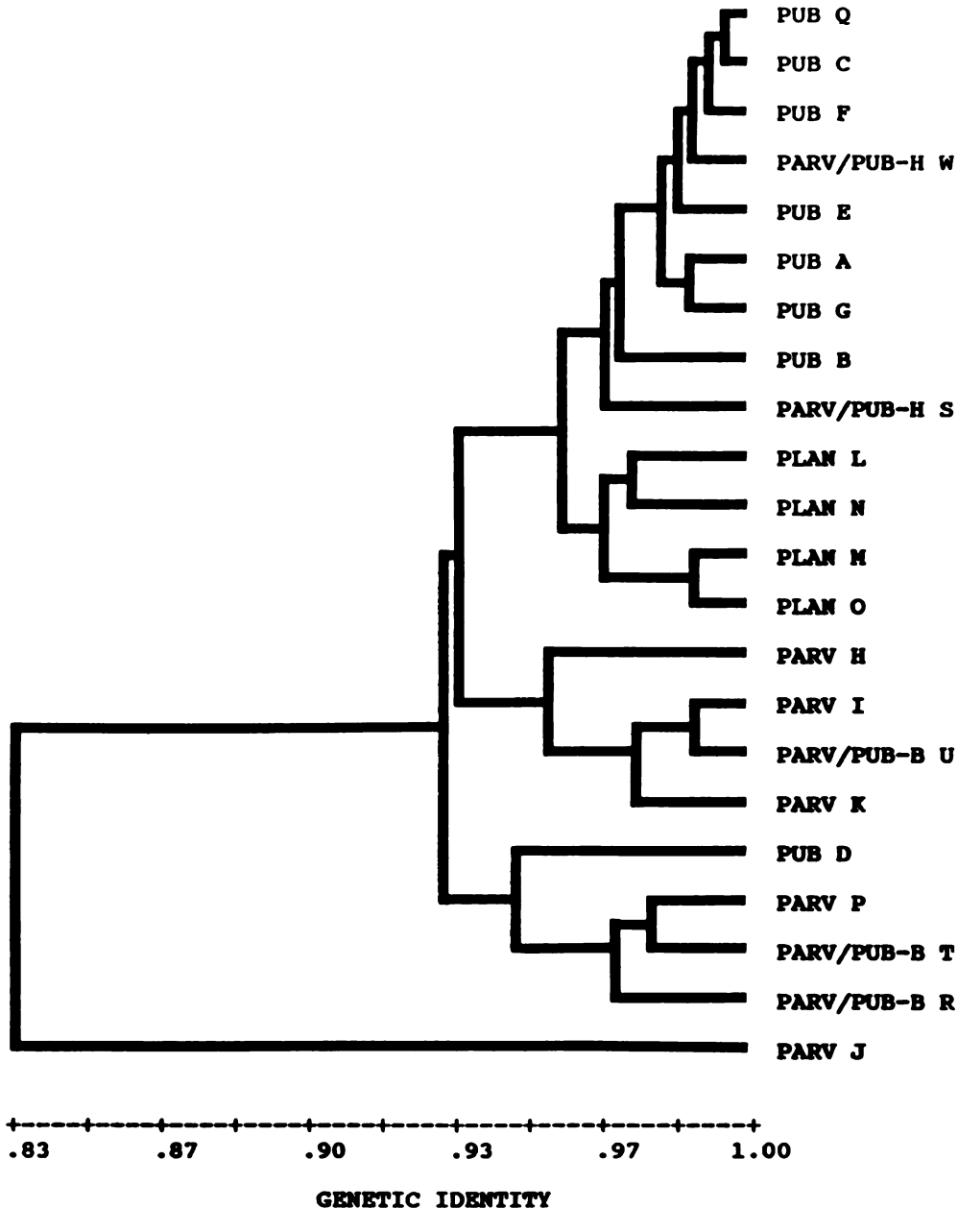
Inter- and intravarietal values of Nei's genetic identities were similar. The means for intravarietal population comparisons ranged from 0.920 to 0.979, and the intervarietal comparisons ranged from 0.900 to 0.954 (Table 2.4). The lowest value occurred between a parviflorum-pubescens comparison (I = 0.747) whereas the highest value was among two populations of var. pubescens (I = 0.999). The UPGMA revealed that populations of var. pubescens and var. planipetalum tended to cluster on separate branches of the phenogram (Figure 2.1). Population D was the only pubescens population that did not cluster with the others. Although parviflorum populations also tended to group together, these populations have a large variance in their genetic identity values. For example, population J and the remaining populations have an average identity value of only 83% compared to the next highest clustering value of approximately 93%. Despite this relatively low genetic identity with other populations, no apparent morphological or ecological aberrations were observed in population J. In fact, it was morphologically and ecologically very similar as well as geographically close to parviflorum population K (Table 2.1). Similarly, no significant geographic association with genetic distance was found for populations of var. parviflorum (rs = -0.31; p

TABLE 2.4. Nei's genetic identity means and ranges for intra- and intervarietal comparisons of C. calceolus.

Comparison	Mean	Range
Intravarietal:		
PUB	0.978	0.952 - 0.999
PARV	0.920	0.839 - 0.988
PLAN	0.979	0.971 - 0.995
MEAN	0.959	0.839 - 0.999
Intervarietal:		
PARV/PUB	0.900	0.747 - 0.980
PARV/PLAN	0.900	0.842 - 0.963
PUB/PLAN	0.954	0.887 - 0.984
MEAN	0.918	0.747 - 0.984

FIGURE 2.1. UPGMA phenogram of Nei's genetic identity values for all pairwise population comparisons of pure var. pubescens (PUB), var. planipetalum (PLAN), var. parviflorum (PARV), and mixed populations (indicated by two-taxon abbreviations separated by a slash). In the mixed populations, an H indicates populations that are only composed of morphologies intermediate to the extreme manifestations of the varieties, whereas a B represents populations with both varieties and intermediates present. The last letter is a key to each population referenced in Table 2.1.




PUB Q
PUB C
PUB F
PARV/PUB-H W
PUB E
PUB A
PUB G
PUB B
PARV/PUB-H S
PLAN L
PLAN N
PLAN M
PLAN O
PARV H
PARV I
PARV/PUB-B U
PARV K
PUB D
PARV P
PARV/PUB-B T
PARV/PUB-B R
PARV J
.83
.87
.90
.93
.97
1.00
GENETIC IDENTITY

> 0.1), or populations of var. planipetalum (rs = 0.23; p > 0.1). A significant positive correlation was found for var. pubescens (rs = 0.50; p < 0.025) but this result should be interpreted with caution. The removal of populations B, C, D, F, or G cause this correlation to become nonsignificant. Similar to var. parviflorum populations, geographically close pubescens populations (e.g., populations A and B; Table 2.1; Figure 2.1) do not necessarily have the closest genetic identities.

The mixed populations are scattered throughout the phenogram (Figure 2.1). Populations with only hybrid morphologies present (i.e., populations W and S) clustered with var. pubescens whereas populations with both varietal forms and intermediates present (i.e., populations U, T, and R) clustered with either pure parviflorum or mixed populations. Population Q (a pubescens population which is sympatric with P) clustered with var. pubescens. Population P (var. parviflorum) clustered in a group of mixed populations which also included the one pubescens outlier population. The phenogram had a cophenetic correlation coefficient of 0.868 and a percent standard deviation of 2.39.

Principal components analysis revealed a similar dispersion of pure populations (Figure 2.2). Populations of var. pubescens formed the tightest cluster and population D was the most divergent of all populations within this variety. Likewise, populations of var. planipetalum tended

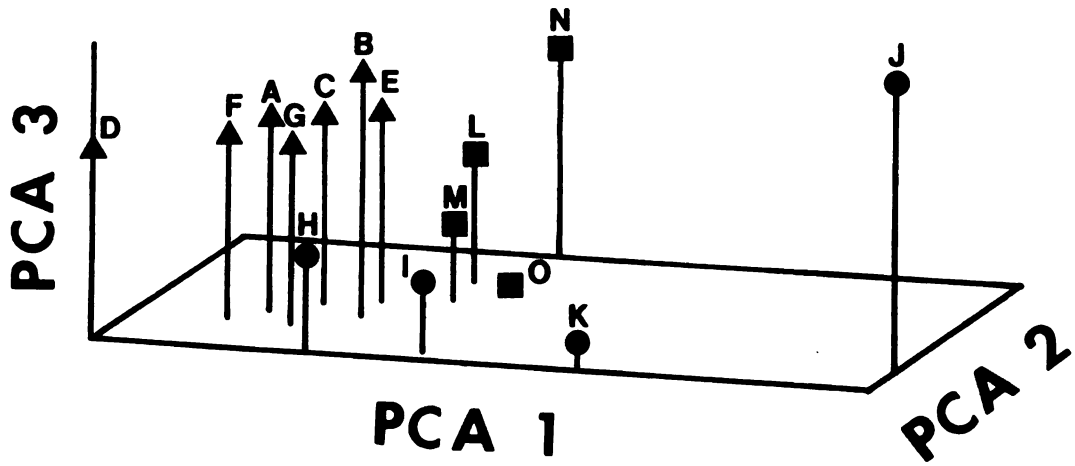


FIGURE 2.2. Pure populations of vars. pubescens (triangles), parviflorum (circles), and planipetalum (squares) plotted in relationship to the first three principal component axes (PCA1, PCA2, and PCA3) of a principal components analysis of allele frequencies. The first three axes together account for 66% of the total variation in allele frequencies. Population letters refer to populations listed in Table 2.1.

to cluster together whereas populations of var. parviflorum showed the highest level of dispersion. Population J was the most divergent population as it was with UPGMA. The principal component axis 1 (PCA 1) is most highly correlated with variation at TPI-2, PGD-2, and IDH-2 and represents 33.28% of the total variation (Table 2.5). Axes 2 and 3 represent an additional 21.31% and 11.75% of the total variation, respectively. Axis 2 is most highly correlated with variation at PGM-1a, PGM-1d, and SKD-1a, whereas axis 3 has the highest loadings from PGM-1b, GOT-2a, PGD-2a, SKD-1c, and SKD-d. The first seven eigenvalues were greater than one and represent 90.3% of the total variation.

For the vast majority of single-locus contingency table analyses, significant allele-frequency heterogeneity ($p < 0.05$) was found for comparisons within and among the varieties (PGD-2 was the only locus that did not show a significant mean difference among the varieties). Within var. planipetalum, four loci did not show significant differences among populations in allele frequencies (i.e., TPI-2, PGM-2, ADH, and PGD-2). The only other locus that did not display significant heterogeneity was ADH in var. parviflorum. Four loci showed significant allele frequency heterogeneity among the sympatric populations P and Q (Table 2.6). Most loci in most populations did not show significant deviations ($p < 0.05$) from Hardy-Weinberg expectations. In pure populations, significant deviations occurred only in five cases distributed across three

TABLE 2.5. Allelic correlations with the first, second, and third principal components, and the percent of variation accounted for by each component (percent trace).

Allele	Principal Component 1	2	3
TPI-1b	0.592	-0.447	-0.237
TPI-1c	-0.592	0.447	0.237
TPI-2a	-0.860	0.284	0.016
TPI-2b	0.860	-0.284	-0.016
PGM-1a	0.094	0.766	-0.305
PGM-1b	0.647	-0.221	0.529
PGM-1c	-0.486	-0.674	-0.080
PGM-1d	0.082	0.835	-0.062
PGM-2a	0.727	0.286	-0.012
PGM-2b	-0.727	-0.286	0.012
GOT-2a	0.659	0.012	-0.542
GOT-2b	0.705	-0.261	0.477
GOT-2c	-0.784	0.050	0.396
ADH-a	-0.656	-0.401	0.300
ADH-b	0.668	0.368	-0.318
ADH-c	0.329	-0.293	-0.431
ADH-d	-0.214	0.002	0.142
ADH-e	0.082	0.602	0.184
PGD-2a	0.065	0.348	-0.519
PGD-2b	0.445	0.544	0.182
PGD-2c	0.829	-0.347	0.048
PGD-2d	-0.464	-0.545	-0.155
IDH-2a	-0.831	0.122	-0.476
IDH-2c	0.832	-0.124	0.476
SKD-a	0.003	-0.899	-0.262
SKD-c	-0.045	0.718	0.602
SKD-d	0.080	0.490	-0.595
Percent Trace	33.28	21.31	11.75

TABLE 2.6. Results from a chi-square contingency table analysis for allele frequency heterogeneity among varieties parviflorum (population P) and pubescens (population Q) at a sympatric site on Drummond Island. Reported here are the four loci that show significant heterogeneity, their allele frequencies, and associated p values for each significant locus.

Locus and Allele	PARV	PUB	p
TPI-2a	0.475	0.925	
TPI-2b	0.525	0.075	< 0.000
GOT-2a	-	0.350	
GOT-2b	-	0.100	
GOT-2c	1.000	0.550	< 0.000
PGD-2b	0.525	0.800	
PGD-2d	0.475	0.200	0.009
SKD-a	0.525	0.275	
SKD-c	0.350	0.600	
SKD-d	0.125	-	
SKD-e	-	0.125	0.001
All Loci Tested			< 0.000

populations and three loci. These have been reported elsewhere (M. Case, Part 1). Only one additional deviation was found (PGM-2 in population R).

DISCUSSION

As seen in Table 2.4, only a slight difference was found between the average intravarietal (I = 0.959) and average intervarietal (I = 0.918) genetic identity values. This indicates that, on average, populations within a variety are only slightly more genetically similar than populations belonging to different varieties. There is, however, considerable overlap in identity values. For example, the mean genetic identity of pubescens-planipetalum comparisons is higher (I = 0.954) than the mean genetic identity of populations within var. parviflorum (I = 0.920). The relatively large variance in genetic identities among parviflorum populations can be visualized in the UPGMA phenogram (Figure 2.1) as well as in a plot of these populations on the first three principal component axes (Figure 2.2). For both techniques, pure populations of var. parviflorum (populations H through K) show a high level of dispersion. Most striking is population J which was geographically close to and morphologically and ecologically very similar to population K. This large difference was due, in part, to IDH-2 which had an uncommon allele in high frequency in population J, and also due to TPI-2, PGM-1b, and PGM-2, all of which displayed aberrant allele

frequencies in this population (tables of populational allele frequencies are available from the author upon request). In contrast, there was substantially less intravarietal differentiation among populations of vars. pubescens and planipetalum. Population D is a notable exception to this trend since it contained frequencies that were unlike other pubescens populations at GOT-2c, ADH-b, and PGD-2b.

The overall genetic identity values among the varieties are high in comparison to the mean value of 0.67 commonly reported for congeneric taxa (Crawford 1983; Gottlieb 1977, 1981b). Furthermore, these identity values are very high when compared to identity values reported for other Cypripedium taxa (I = 0.00 - 0.79; M. Case, Part 1). However, these values are typical of those reported for conspecific populations (Gottlieb 1977) including population comparisons among infraspecific taxa (Crawford and Smith 1984). For example, Crawford and Smith (1984) found that four morphologically variable varieties of Coreopsis grandiflora Hogg ex Sweet exhibited intravarietal values which ranged from 0.81 to 0.99 and intervarietal values which similarly ranged from 0.79 to 0.99. A similar lack of allozyme divergence among infraspecific taxa has also been documented for taxa within Ipomopsis aggregata (Pursh) V. Grant (Wolf et al. 1991), Gaillardia pulchella Foug. (Heywood and Levin 1984), and others reviewed in Crawford and Smith (1984). In contrast, relatively low genetic

identities have previously been documented for some other infraspecific taxa. In varieties of Allium douglasii Hook. (Rieseberg et al. 1987) and Coreopsis cyclocarpa Blake (Crawford and Bayer 1981), mean intervarietal genetic identities were 0.84 and 0.75, respectively, compared to mean intravarietal values of over 0.95 for both species. Even lower values were found among ecotypes of the orchid Gymnaedenia conopsea (L.) R. Br. (Scacchi and De Angelis 1989). Typical conspecific identity values of 0.98 and 0.96 were found within a dry and wet ecotype of this species, respectively, whereas the average interecotype genetic identity was only 0.65. Although infraspecific taxa can exhibit a wide range of genetic identity values, low values are most commonly associated with either allopatric or ecologically isolated taxa (Heywood and Levin 1984; Rieseberg et al. 1987) as in the above examples. This result suggests, as Heywood and Levin (1984) have discussed, that large divergence in allozyme frequencies occurs predominantly after the severance of gene flow among taxa (i.e., after "speciation" as defined by Heywood and Levin 1984). Therefore, the lack of large allozyme differences among Cypripedium varieties is typical of sympatric infraspecific taxa which experience gene flow.

The lack of ubiquitous, moderate- to high-frequency unique alleles within these varieties of Cypripedium (Table 2.3) is another indication that they are genetically similar and have recently experienced gene flow. The differences that

contributed to the structure in the phenogram were allele-frequency differences among the varieties. Although there is often great overlap in frequencies, the alleles at TPI-1, and GOT-2c showed the greatest mean differences among parviflorum and pubescens populations. Other moderate differences were found between vars. parviflorum and planipetalum at SKD-a and TPI-1, and at PGM-1c for vars. pubescens and planipetalum (Table 2.7). The overall similarities of these taxa is further reflected by the hierarchical F-statistics which revealed that the largest amount of variation within the species is contained within individual populations (81%). Of the variation that is distributed among populations, 12% was found within varieties and the smallest portion (7%) was distributed among varieties.

Although the general lack of large allozymic differences among the varieties creates some ambiguity with the interpretation of the mixed populations, the phenogram (Figure 2.1) suggests several trends. The first involves the status of populations W and S. These populations consisted entirely of plants with morphologies intermediate to the extreme manifestations of vars. parviflorum and pubescens. According to the concept of Case (1987) these plants fit well within the variation described for var. pubescens, which is generally considered to be more morphologically variable than var. parviflorum. The allozyme data also suggest that these populations with

TABLE 2.7. Allele-frequency means for polymorphic loci (weighted according to population sizes), population ranges, and number of individuals sampled for varieties of C. calceolus.

Locus and Allele	Taxon and Number of Individuals Sampled		
	PARV n = 207	PUB n = 140	PLAN n = 78
TPI-1b	0.572 (0.295 - 0.676)	0.093 (0.000 - 0.350)	0.186 (0.025 - 0.306)
TPI-1c	0.428 (0.324 - 0.705)	0.907 (0.650 - 1.000)	0.814 (0.694 - 0.975)
TPI-2a	0.614 (0.298 - 0.835)	0.907 (0.675 - 1.000)	0.821 (0.750 - 0.875)
TPI-2b	0.386 (0.165 - 0.702)	0.093 (0.000 - 0.325)	0.179 (0.125 - 0.250)
PGM-1a	0.002 (0.000 - 0.010)	- -	0.308 (0.200 - 0.500)
PGM-1b	0.114 (0.000 - 0.489)	0.057 (0.000 - 0.175)	- -
PGM-1c	0.884 (0.511 - 1.000)	0.940 (0.825 - 1.000)	0.577 (0.389 - 0.750)
PGM-1d	- -	0.003 (0.000 - 0.025)	0.115 (0.050 - 0.225)
PGM-2a	0.266 (0.108 - 0.511)	0.186 (0.050 - 0.450)	0.295 (0.222 - 0.350)
PGM-2b	0.734 (0.489 - 0.892)	0.814 (0.550 - 0.950)	0.705 (0.650 - 0.778)
GOT-2a	0.582 (0.375 - 0.850)	0.261 (0.025 - 0.475)	0.532 (0.333 - 0.700)
GOT-2b	0.053 (0.000 - 0.202)	0.014 (0.000 - 0.100)	- -

Table 2.7 (cont'd)

GOT-2c	0.365	0.725	0.468
	(0.150 - 0.625)	(0.525 - 0.975)	(0.300 - 0.667)
ADH-a	0.022	0.114	-
	(0.000 - 0.045)	(0.025 - 0.250)	-
ADH-b	0.976	0.882	0.994
	(0.955 - 1.000)	(0.750 - 0.975)	(0.975 - 1.000)
ADH-c	0.002	-	-
	(0.000 - 0.010)	-	-
ADH-d	-	0.004	-
	-	(0.000 - 0.025)	-
ADH-e	-	-	0.006
	-	-	(0.000 - 0.025)
PGD-2a	-	-	0.006
	-	-	(0.000 - 0.025)
PGD-2b	0.841	0.861	0.917
	(0.682 - 0.979)	(0.550 - 1.0000	(0.875 - 1.000)
PGD-2c	0.005	-	-
	(0.000 - 0.011)	-	-
PGD-2d	0.154	0.139	0.077
	(0.011 - 0.318)	(0.000 - 0.450)	(0.000 - 0.125)
IDH-2a	0.780	0.964	0.942
	(0.245 - 0.983)	(0.775 - 1.000)	(0.800 - 1.000)
IDH-2c	0.220	0.036	0.058
	(0.017 - 0.755)	(0.000 - 0.225)	(0.000 - 0.200)
SKD-a	0.606	0.368	0.109
	(0.372 - 0.773)	(0.175 - 0.625)	(0.000 - 0.200)
SKD-c	0.394	0.632	0.712
	(0.227 - 0.628)	(0.375 - 0.825)	(0.425 - 0.975)
SKD-d	-	-	0.179
	-	-	(0.025 - 0.425)

intermediate-looking plants have the genetic constitution of var. pubescens. It should be noted, however, that individuals in these populations were morphologically uniform as compared to other "mixed" populations where both varieties were present in addition to plants with intermediate morphologies. In the latter case, the intermediates often display much morphological variation. This is the situation in populations U, T, and R, which consisted of extreme morphologies of var. parviflorum and var. pubescens in addition to a continuum of forms between the extremes. All of these populations clustered away from the majority of pubescens and were most like other parviflorum populations or other mixed populations of the same type. This is suggestive of a genetic influence from var. parviflorum. Some of the most interesting results in this study concern the sympatric populations P and Q. Individuals from these populations appeared to be randomly intermixed in the field with no indication of morphological intermediacy. In the phenogram, they each cluster in the vicinity of their respective morphological varieties (Figure 2.1), and they display significant allele-frequency differences at four loci (Table 2.6). This result is highly suggestive of restricted gene flow between populations P and Q and supports the observations of Newhouse (1976), Atwood (1985), Case (F. Case, pers. comm.), and others that, in some geographic areas, these two varieties display characteristics typical of species. Investigation of

population structure and evolutionary processes in these and other populations of vars. pubescens and parviflorum are ongoing.

All varieties of C. calceolus contain species-level expected heterozygosity values which are higher than the average reported for other plant species (Ht = 0.149; Hamrick and Godt 1989), and generally higher or very near the average values reported for plants with either a widespread distribution (Ht = 0.20), long-lived herbaceous perennial habit (Ht = 0.20), or an animal-outcrossed mating system (Ht = 0.16; Hamrick and Godt 1989). Although a high level of genetic variation accompanies a large level of morphological variation in this species, the taxon often cited to have the most morphological variation (i.e., var. pubescens) is the taxon with the lowest overall level of genetic variation (Ht = 0.18; Table 2.2). Another difference among the taxa is the proportion of variation that is distributed among populations. Varieties pubescens and planipetalum populations have similar Gst values of 0.084 and 0.053, but var. parviflorum populations are approximately twice as structured with a Gst value of 0.171 (Table 2.2). Hamrick and Godt (1989) found that a long-lived perennial habit and an animal-outcrossed pollination system were the two traits which accounted for the largest proportion of variation in Gst values in plant species. However, since varieties of C. calceolus do not differ qualitatively for these traits, this is an unlikely

explanation for the observed intervarietal variation in Gst values. One hypothesis which could account for this variation is that there are differences among the taxa in the amount of intravarietal gene flow. Since var. parviflorum is most often associated with wet environments (Case 1987) and is more ecologically restricted than var. pubescens, it is likely that parviflorum populations are more geographically isolated and therefore occur in more of a mosaic pattern than populations of var. pubescens, which occupy a diverse array of habitats. Genetic drift could act to increase genetic variance among parviflorum populations relative to pubescens populations which would be reflected in higher Gst values for var. parviflorum. Likewise, the relatively high degree of population similarity among planipetalum populations could be due to higher levels of intravarietal gene flow for this taxon. The occurrence of neighboring populations of var. planipetalum in close proximity on treeless, windswept, limestone barrens in Newfoundland could produce higher levels of intravarietal gene flow relative to the more geographically-isolated populations of var. parviflorum.

Although var. parviflorum has a higher Gst value than either var. pubescens or var. planipetalum, var. parviflorum is most similar to the averages reported for either long-lived herbaceous perennials or animal-outcrossed plants (Gst = 0.213 and 0.197, respectively; Hamrick and Godt 1989). Moreover, the Gst values for var. planipetalum and var.

pubescens are substantially lower than these mean values. This could be due to a common origin of the study populations within each of these varieties. Since all of the populations in the present study are either north of or very close to the southernmost border of the Wisconsin continental ice sheet (Péwé 1983), plants have probably colonized these areas within the last 11,000 years (Webb et al. 1983). It is possible that a rapid radiation from a single source population into the geographic regions examined in the present study could produce populations which are genetically similar. This has been proposed for other Cypripedium species in addition to taxa in other genera (see review in M. Case, Part 1).

It is evident that Cypripedium calceolus is composed of populations which have a great potential to evolve with high levels of heterozygosity and regional variation in the tendency to form hybrids. In the northeastern United States and southeastern Canada, small levels of genetic divergence were found among vars. pubescens, parviflorum, and planipetalum, and slight differences were observed in levels of heterozygosity and distribution of genetic variation among populations within these taxa. These differences suggest that multiple taxa exist. However, the lack of high-frequency novel alleles in any of the varieties, the large overlap in allele frequencies among the varieties, and the large variance in the allele frequencies found in var. parviflorum prevent definitive classification of these taxa

through allozyme data. Because of this and the general agreement of these results with other previously-reported allozyme surveys of sympatric infraspecific taxa, it is suggested that vars. parviflorum, pubescens, and planipetalum be considered members of a single species.

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