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Systematic and Population Genetic Analyses of Northern Vs Southern Yellow Lady's Slippers (Cypripedium parviflorum Vars parviflorum, pubescens, and makasin): Inference from Isozyme and Morphological Data

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SYSTEMATIC AND POPULATION GENETIC ANALYSES OF NORTHERN VS. SOUTHERN YELLOW LADY'S SLIPPERS

(Cypripedium parviflorum vars. *parviflorum, pubescens,* and *makasin)*: INFERENCE FROM ISOZYME AND MORPHOLOGICAL DATA

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By

Lisa E. Wallace

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of the requirement for the degree of

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ABSTRACT

Cypripedium parviflorum is a wide-ranging North American orchid species that contains high levels of morphological and genetic variability, as well as variation in population isolating mechanisms. This complexity has fostered considerable taxonomic debate over the number of taxa that may exist in the species, and also the taxonomic level at which these taxa should be recognized. A recent isozyme analysis by Case (1993) demonstrated that two varieties should be recognized within the species, *Cypripedium parviflorum* var. *parviflorum* and *C. parviflorum* var. *pubescens*. However, her study was based on the examination of populations in Michigan and surrounding states, which represents a relatively small portion of the entire species' range.

Recently, it has been proposed by Sheviak (1994) that three distinct varieties of the species exist. While the concept of var. *pubescens* does not change in Sheviak's treatment, var. *parviflorum* is now interpreted to be restricted to the southeastern United States, and var. *makasin* is considered to be a northeastern taxon. This thesis examines Sheviak's hypothesis and extends the work of Case. Populations representing all three taxa and extending from Northern Michigan to Georgia are examined via morphological and isozyme analyses. Specifically, the following questions are addressed: 1) Do the morphological and isozyme data support the distinction and recognition of three varieties within *C. parviflorum*?, 2) Can the conclusions reached in prior isozyme analyses of northern *C. parviflorum* populations be extended to southern populations of this taxon?, and 3) Do similar levels of genetic variation exist in northern versus southern populations of *C. parviflorum*? An analysis of 15 morphological characters and protein variation at 13 isozyme loci were conducted to address these questions.

Univariate statistics, principal components analysis (PCA), and unweighted pair group methods analysis (UPGMA) of morphological characters show that vars. *parviflorum* and *makasin* are largely indistinguishable from one another. However, these varieties are morphologically distinct from southern as well as northern populations of var. *pubescens*. This result suggests that vars. *makasin* and *parviflorum* should be considered the same taxonomic entity, which does not support the Sheviak hypothesis. A different outcome was obtained with the isozyme data. In UPGMA of Nei's Genetic Identity based on population allele frequencies, populations of var. *makasin* cluster away from vars. *pubescens* and *parviflorum*, but the latter two taxa are indistinguishable from each other. This lends support to the hypothesis that var. *makasin* represents a separate genetic identity, although a very large variance among populations of var. *makasin* precludes a definitive delimitation of this taxon based on allele frequency data. Therefore, Sheviak's hypothesis is not generally supported because it is not possible to define var. *makasin* with either morphological or isozyme data. The isozyme data also indicate that: (1) the varieties maintain relatively high levels of genetic diversity [average expected heterozygosity values = 0.157 (var. *parviflorum*), 0.171 (var. *pubescens*), and 0.253 (var. *makasin*)], (2) each variety maintains moderate levels of genetic variation distributed among populations [Nei's Gst = 0.196 (var. *pubescens*), 0.162 (var. *parviflorum*), and 0.265 (var. *makasin*)], and (3) all varieties display general conformance to Hardy-Weinberg equilibria.

The results of this study are largely consistent with previous conclusions based on analyses of northern populations of vars. *pubescens* and *makasin*, but the close isozyme similarity of vars. *pubescens* and *parviflorum* was unexpected. Another unexpected result was the discovery that southern populations of var. *pubescens* and var. *parviflorum* are significantly less variable than their northern counterparts. One hypothesis that could account for these unexpected findings concerns the post-glacial migration history of these taxa. It is suggested that northern areas were particularly suitable for the colonization and maintenance of large populations as glaciers retreated. Southern areas, however, may have been vegetated more heavily, containing habitats less conducive to large population sizes and interpopulation gene flow. This situation would have created a greater loss of alleles due to genetic drift in the south compared to northern areas, and may have produced the relatively close isozyme similarity of vars. *pubescens* and *parviflorum* in the south. Evidence for this hypothesis as well as the conservation implications of it are discussed.

SYSTEMATIC AND POPULATION GENETIC ANALYSES OF NORTHERN vs. SOUTHERN YELLOW LADY'S SLIPPERS (*Cypripedium parviflorum* vars. *parviflorum, pubescens,* and *makasin*): INFERENCE FROM ISOZYME AND MORPHOLOGICAL DATA

INTRODUCTION

The North American Yellow Lady's Slipper, *Cypripedium parviflorum* Salisb. and its associated varieties [i.e., vars. *parviflorum, pubescens* (Willd.) Knight, and *makasin* (Farwell) Sheviak] are diploid (2N=20) outcrossing members of the subfamily Cypripedioideae Lindley (Orchidaceae; Sheviak, 1994; Dressler, 1981). As currently recognized, *C. parviflorum* var. *pubescens* occupies the greatest geographic range, occurring in approximately 40 states (Luer, 1975). *Cypripedium parviflorum* var. *parviflorum*, however, has recently been segregated into two varieties, var. *makasin* (a northern entity occurring largely in Canada and the northeast United States) and var. *parviflorum* (which occurs predominantly in the southeastern half of the United States; Sheviak, 1994). The following discussion of the taxonomic history of this group refers to *Cypripedium parviflorum* var. *parviflorum sensu lato* unless indicated otherwise.

The subspecific classification of *Cypripedium parviflorum* is obscured by the extensive morphological variation it exhibits and the widespread occurrence of intraspecific and interspecific hybrids. Subsequently, it has been the center of great taxonomic controversy for over 200 years (Newhouse, 1976). Salisbury was the first to recognize the North American entity, which he named *C. parviflorum*, as distinct from the Eurasian *Cypripedium calceolus* L., based largely upon differences in staminode shape. In 1804, Willdenow segregated the North American taxon into two species, *C. pubescens* and *C. parviflorum*, citing differences in the lobes the column.

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In 1918, Farwell recognized three distinct Yellow Lady's Slipper taxa based on his observations of size and plane of compression of the slipper, and the shape of the staminode. These were *C. pubescens* var. *makasin, C. pubescens* var. *pubescens,* and *C. parviflorum*. Later, Correll (1938), noting extraordinary variability within the North American plants, recognized all North American plants as morphological variants of *C. calceolus* var. *pubescens*. Fernald (1946), although maintaining *C. calceolus,* chose to split the North American taxa into two varieties, var. *pubescens* and var. *parviflorum*.

Only recently has the North American entity been reassigned to its own species, *C. parviflorum,* separate from *C. calceolus*. Sheviak (1994) reports that intercontinental differences are exhibited most notably in staminode morphology. The North American taxa have staminodes which are conduplicate (folded like an open book), yellow, and broadest at the base or middle. In contrast, the Eurasian species has a canaliculate staminode (trough-like) which is white and broadest near the apex. Sheviak (1994) notes that coloration and broadest point may vary somewhat, but that the canaliculate shape of the staminode is widespread, and most notably segregates *C. calceolus* from *C. parviflorum*. Furthermore, *C. calceolus* is regarded as being less morphologically variable than *C. parviflorum*.

Additionally, Case (pers. comm.) has found dramatic differences in the alleles present among North American and Eurasian species, presumably indicating a degree of genetic relatedness closer to congeneric species than conspecific varieties. In their analysis of floral fragrances, Bergstrom et al. (1992) found distinct differences in the chemical composition of scent among the Eurasian *C. calceolus* and the North American taxa *C. parviflorum* var. *parviflorum* and *C. parviflorum* var. *pubescens*. This evidence lends

support to the delimitation of two species, *C. parviflorum* in North America and *C. calceolus* in Eurasia.

Presently, the controversy involves the taxonomic status of the various North American taxa. Some authors (e.g., Atwood, 1985) maintain the distinction at the specific level (i.e., *C. parviflorum* and *C. pubescens*), citing the presence of reproductive isolating mechanisms evident in sympatric populations in which no intermediate morphologies have been observed. Other authors (e.g., Case, 1993; Sheviak, 1992, 1994) recognize the forms distinct at the varietal level. Notably, Case (1993), using variation at isozyme loci assayed from vars. *parviflorum* and *pubescens*, found estimates of genetic divergence comparable to values among conspecific populations rather than congeneric species. Based upon morphology and geographical distribution, Sheviak (1992, 1994) also supports the existence of varieties. However, all botanists who have studied the species recognize the vast amount of variation contained within the species complex.

Since the original description of *C. parviflorum* by Salisbury, botanists have noted the large levels of morphological and ecological variation that exist throughout the range. Nearly every quantitative and qualitative morphological measurement possible has been studied on these plants with virtually no consensus of diagnostic limits for any of the taxa. For example, measurements of slipper length, a trait used to differentiate varieties, range from 1 cm up to 6.5 cm (Sheviak, 1994; Homoya, 1993; Klier et al., 1991; Summers, 1987; Gupton and Swope, 1986; Newhouse, 1976; Luer, 1975). In addition, plant height has been considered to be highly diagnostic for some taxa, ranging in the complex from 10 cm up to 90 cm (Homoya, 1993; Klier et al., 1991; Summers, 1987; Gupton and Swope, 1986; Luer, 1975). However, plant height may be ecologically variable with the habitat of

the plant [e.g., I observed an inverse correlation of height and exposure to direct sunlight; Sheviak (1992, 1994) reports similar cases of potential phenotypic plasticity.] The varieties may also differ in soil type as var. *parviflorum* tends to occur in drier, often more acidic sites than var. *pubescens,* and var. *makasin* is found in calcareous fens as well as other wet sites with organic rich or sandy soils (Sheviak, 1994). However, other botanists differ on this point citing a range of soil habitats across all varieties (e.g., Correll, 1938; Atwood, 1985; Muik, 1979).

To further complicate matters, the varieties of Yellow Lady's Slippers can hybridize with each other (Harms, 1986; Sheviak, 1992; Stoutamire, 1967) and with congeneric species [e.g., *C. parviflorum* x *C. candidum* Muhl. ex Willd. (Klier et al., 1991) and *C. parviflorum* x *C. montanum* Douglas ex. Lindl. (Sheviak, 1992)], making it difficult to distinguish natural intravarietal variants from hybrids.

Sheviak (1994) interprets all of this morphological and ecological variation as evidence for the existence of several taxa, and subsequently has chosen to recognize three varieties: *C. parviflorum* var. *pubescens*, the large flowered variety; *C. parviflorum* var. *parviflorum*, the southern small flowered variety; and *C. parviflorum* var. *makasin*, the northern small flowered variety. In his published key, Sheviak (1994) uses five characters to discriminate among varieties. These include 1) degree of pubescence on the sheathing bract, 2) slipper size, 3) spotting of the sepals/petals, 4) scent, and 5) geographic range. The variety *makasin* is characterized by: 1) a "sparsely and inconspicuously pubescent to glabrous" sheathing bract in young plants, 2) small flowers with a lip length of 15-29 mm, 3) a suffusion of deep reddish brown or madder coloring on the petals and sepals, 4) an intense, sweet scent, and 5) a geographic range which extends across Canada and the

United States as far south as New England and the Great Lakes. In contrast, both varieties *parviflorum* and *pubescens* can be characterized by a "densely and conspicuously silvery-pubescent" sheathing bract in young plants, as well as a faint, musty smell. However, var. *parviflorum* and var. *pubescens* also exhibit differences in the other characters. The variety *pubescens* has large flowers with a lip length up to 54 mm (although it may be smaller in plants of boreal and northern cordilleran areas), and scattered spots of reddish brown or madder on the sepals and petals. The range of this variety encompasses the ranges of both of the other varieties, extending across North America and, in the eastern half of the United States, as far south as Louisiana [Sheviak is in agreement with the range presented by Luer (1975) for var. *pubescens*]. The variety *parviflorum* has small flowers with a lip length of 22-34 mm, and densely spotted reddish brown or madder on the sepals and petals. The range of this variety is from southern New England west to Kansas and southward to Louisiana (Sheviak, 1994).

Due to the high levels of morphological and ecological variation and an historical difficulty in the delimitation of varieties based upon morphological characters, alternative analyses have been explored in order to resolve the dispute over taxonomic rank and taxa delimitation. Isozyme electrophoresis has proven to be a powerful technique at lower taxonomic levels (e.g., below genera; Gottlieb, 1977; Schall et al, 1991). It has the advantage of providing detailed genetic analyses of populations without the hindrance of environmental factors influencing the variables that morphological data are subject to (Gottlieb, 1977). Results obtained from isozyme electrophoresis provide estimates of the distribution and abundance of genetic variation within and among populations. Furthermore, this analysis is applicable to questions of systematic interest because

estimates of genetic relatedness among taxa are possible.

Thus far, there is no quantitative information available on the relationship between genetic variation, morphological variation, and geographic distribution for this species. Case (1993, 1994) has demonstrated that the *C. parviflorum* species complex exhibits unusually high levels of variation. However, she did not sample from the southern part of the species range (i.e., from south of Ohio), and no morphometric analyses were included in her data. On the other hand, Sheviak (1992, 1994) has thoroughly documented the morphology, ecology, and geographic distribution of the species complex, but he has not included genetic data or statistical analyses of the variation in morphological characters. Therefore, in this study, I seek to integrate morphological variation with genetic variation throughout a large portion of the species range in the eastern United States.

The results of isozyme electrophoresis are presented in comparison to and in conjunction with a re-examination of Case's data and new morphological data. This enables Case's work to be extended and re-evaluated with the inclusion of populations in the southeastern United States. Furthermore, Sheviak's proposal of a new classification is evaluated based upon divergence at isozyme loci. Specifically, the study addresses the following questions: 1) Does the quantitative morphological and/or isozyme data support the distinction and recognition of three varieties within the *C. parviflorum* species complex? 2) Can the conclusions reached in prior isozyme analyses of northern *C. parviflorum* populations be extended to southern populations of this taxon? 3) Do similar levels of genetic variation exist in northern versus southern populations of *C. parviflorum*?

MATERIALS AND METHODS

Populations Studied

Populations chosen for sampling in the present study were located throughout the eastern United States (Fig. 1). Taxa were identified in the field based upon Sheviak's (1994) concepts of morphological character, geographical, and habitat descriptions for each of the varieties *parviflorum, pubescens,* and *makasin*. Two populations (B, G; Table 1) were not in flower at the time of sampling. These populations were given varietal status based upon historical records of taxonomic status. A total of 30 pure and mixed populations are included in this study (Table 1). Pure populations are defined as those containing only one variety, clearly distinguishable from any other variety (pure *pubescens* populations: A-N; pure *parviflorum* populations: O-U; pure *makasin* populations: V-W). Mixed site populations were of two types: I) populations with some individuals displaying intermediate morphologies while others being clearly distinguishable into one of the three varieties (listed as BOTH in Table 1), and II) populations in sympatry in which two varieties were clearly present, and no intermediate morphologies were found (SYM). Only one population (X) fits into the type I category while six populations (Y, Z, AA, BB, CC, DD) are of type II.

Although every effort was made to locate populations of 20 or more genets, some populations consisted of fewer than 20 genets. Population sizes (i.e., the number of stems) ranged from approximately eight plants up to 1000 or more plants. Voucher

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specimens were collected from most populations, and are deposited in the William and Mary herbarium (WILLI).

Morphological Analysis

From the populations in flower, 92 individuals from 27 populations (Table 1) were randomly selected for morphological analysis. This included 43 individuals of the variety *parviflorum*, 42 individuals of the variety *pubescens*, and seven individuals of the variety *makasin*. For each individual, the following characters were measured: (1) plant height from the ground to the tip of the dorsal sepal, (2) number of twists per lateral petal, (3) staminode length, (4) leaf length, (5) leaf width of the largest leaf, (6) petal length, (7) petal width, (8) dorsal sepal length, (9) dorsal sepal width, (10) lateral sepal length, (11) lateral sepal width, (12) slipper length, (13) slipper width, (14) orifice length, and (15) orifice width (Appendix 1). All widths were measured at the widest point, and all measurements except the number of twists per lateral petal are in centimeters. A subset of these characters has been used by Sheviak (1994) to distinguish between varieties *parviflorum* and *makasin*. Klier et al. (1991) also included these and other measurements in their genetic analysis of *Cypripedium candidum*, *C. pubescens*, and associated hybrids. Qualitative characters including scent, slipper color and overcolor, flowering status, and life stage (e.g., juvenile, adult) were also recorded for all individuals sampled. Although these characters were not included in statistical analyses, they facilitated classification in the field.

The arithmetic mean, range, and standard error for each trait were calculated for each variety. Due to unequal variances among the groups, a non-normal distribution of the variates, and small sample size of the *makasin* group, an analysis of variance was not

applicable. Therefore, nonparametric tests were used to test for significant differences in

morphology among the varieties. Ignoring population boundaries, each individual was

placed into one of three groups: *pubescens* (PUB), *parviflorum* (PARV), or *makasin*

(MAK) based upon classification in the field. A Kruskal-Wallis nonparametric rank test

(Sokal and Rohlf, 1995) was performed on the entire data set to evaluate overall

significant differences for each character. Subsequently, Dunn's nonparametric multiple

comparisons test (Zar, 1996) was utilized for the characters found to be significantly

different ($p \leq 0.05$) in the Kruskal-Wallis test. Dunn's test provided for further evaluation

to determine if a variety was significantly different from any other variety for any given

character.

The morphological characters were also subjected to principal components analysis

(PCA) using NTSYS-pc (Rohlf, 1988) to explore if any natural groupings exist in the

data. Furthermore, this analysis was also used to explore if any natural groups correspond

to the geographical or varietal status of individuals resulting from classification based on

morphology. All individuals were included in this analysis. In this and all subsequent

principal component analyses based on morphology, the variates were standardized by

transforming them into units of standard deviation from the mean (NTSYS-pc; Rohlf,

1988). The first three principal component axes were extracted and the individuals were

plotted. An analysis based on all characters, vegetative and flower, yielded no apparent

groupings of individuals. Therefore, the vegetative characters and flower characters were

separated into two data sets, and PCA was performed again on each set. All individuals

were used in each of these analyses. Similarly, the first three principal component axes

were extracted and the individuals were plotted. In an effort to compare the relationships

among populations based on morphological traits, PCA was also performed on mean

population values for each of the characters. Three principal components were extracted

and the populations were plotted. In addition to PCA, unweighted pair group method

analysis (UPGMA) based on Average Taxonomic distance, Euclidean distance, and

Manhattan distance of mean population morphological characters was used to assess the

degree of morphological similarity among populations.

Isozyme Analysis

A total of 515 individuals representing 30 populations were sampled for enzyme

electrophoresis. In populations of fewer than 20 genets, all individuals were sampled. In

populations of 20 or more individuals, a random sampling was conducted in which 20-40

leaf samples were taken. Individuals which were included in the morphological analysis

were also sampled for isozyme electrophoresis.

Many populations contained clumps of individuals suspected of being clonally

produced. Due to the inability to definitively determine (i.e., without physically digging up

the plants) if a clump was clonally produced, several members of the clump were sampled.

By determining the multilocus genotype of each individual in a clump, I determined which

individuals appeared to be clonally produced. In clumps where multiple individuals shared

the same multilocus genotype they were assumed to be clones of each other. In these

situations, one individual from each unique genotype was represented in analyses.

For each population both flowering and non-flowering individuals were sampled.

Additionally, plants at all stages (i.e., juveniles and adults) were included in sampling.

From each plant sampled, a small piece of leaf tissue (ca. 3 cm^2) was taken, divided in

half, and each half was placed in a 1.5 ml centrifuge tube. Samples were kept on ice in the

field and later frozen at -76° C in the laboratory until they were processed. Leaf tissue was ground in a Tris-HCl extraction buffer (Gottlieb, 1981a) using cold (4° C) mortars and pestles. From the homogenized extract, wicks were dipped and stored at -76° C until assayed on a gel. Tissue extracts were prepared no more than one week before being electrophoresed. Twelve percent starch gels were used in combination with three buffer systems which enabled the resolution of 11 enzyme systems. Glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1), triosephosphate isomerase (TPI, E.C. 5.3.1.1), alcohol dehydrogenase (ADH, E.C. 1.1.1.1), glutamate dehydrogenase (GDH, E.C. 1.4.1.2) phosphoglucoisomerase (PGI, E.C. 5.3.1.9), and superoxide dismutase (SOD, E.C. 1.15.1.1) were resolved on a lithium- borate system (Crawford, 1982). A histidine system was used to resolve malate dehydrogenase (MDH, E.C. 1.1.1.40), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), shikimate dehydrogenase (SKD, E.C. 1.1.1.25), and phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44) (Gottlieb, 1981a). Phosphoglucomutase (PGM, E.C. 5.4.2.2) was resolved on a sodium-borate system (Crawford, 1982). All enzyme systems except GOT, ADH, GDH, and SOD were stained using the agarose overlay procedures described by Soltis et al. (1983) with slight modifications. The staining protocol for GOT followed that outlined by Crawford (1982). ADH, GDH, and SOD were all resolved on the same slice using a stain bath (Soltis et al., 1983) with the addition of 5 ml of 100% ethanol in order to visualize ADH.

Gel slices were scored as soon as bands could be visually distinguished. Based on established reports of the quaternary structure of the enzymes assayed as well as the minimum number of isozymes present, genotypes were determined directly from the enzyme phenotypes displayed on the gels (Gottlieb, 1981b; Weeden & Wendel, 1989).

Allozymes could be distinguished from isozymes based on previous work by Case (1993, 1994). The fastest anodally migrating locus was designated 1, the second fastest 2, and so on until all loci were numbered. Similarly, alleles at each locus were given alphabetic designations with the fastest migrating allele named a, and successively slower alleles b, c, etc. Proteins suspected of having similar mobilities across populations were verified by running individuals side by side on the same gel. Allele frequencies were calculated for all populations and for each variety weighted according to population sample sizes. Due to the possibility of introgression between individuals of the mixed population (X), this population was not included in calculations of varietal allele frequencies.

Several measures of diversity were calculated. These include the number of alleles per locus (A), percent polymorphic loci (P), observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}) based on Hardy-Weinberg equilibrium, and the diversity statistics of Nei (1973) and Wright (1984). The number of alleles per locus was calculated for each population (A_P), each variety (A_V), and the species as a whole (A_S). The number of alleles per locus in all cases was calculated by dividing the total number of alleles present by the total number of loci assayed. Similarly, the percent polymorphic loci was calculated at the population (P_P), varietal (P_V), and species (P_S) levels by dividing the number of loci with two or more alleles by the total number of loci assayed. Observed versus expected values of heterozygosity were computed for each population. Additionally, each locus in each population was tested for deviations from Hardy-Weinberg equilibrium using the program BIOSYS-1 (Swofford and Selander, 1989). In this analysis, expected genotypic frequencies were calculated using Levene's (1949) correction for small sample size. Exact significance probabilities were calculated whereby all genotypes in multi-allelic loci (i.e., >

2) were pooled into three classes when expected frequencies of some genotype classes were low. Elston and Forthofer (1977) argue that exact significance probabilities more accurately reflect significant deviations from Hardy-Weinberg equilibrium than tests that use the X^2 distribution.

Wright's hierarchical F-statistics (Wright, 1984) and Nei's diversity statistics (Nei, 1973) were calculated using BIOSYS-1 (Swofford and Selander, 1989). For Wright's hierarchical F-statistics, the amount of genetic variation in the species as a whole was partitioned into three components: the genetic diversity within populations, the genetic diversity among populations within varieties, and the amount of genetic diversity among varieties. These estimates were expressed as percentages of the total variation. For Nei's diversity statistics, **Ht = Hs + Dst**, where Ht is the total expected heterozygosity across all populations, Hs is the average expected heterozygosity within populations, and Dst is the amount of variation distributed among populations. The proportion of genetic variation distributed among populations (Gst) relative to the total expected heterozygosity was calculated from the equation **Gst = Dst/Ht**. Nei's diversity statistics were calculated for each variety and also at the species level.

Although BIOSYS-1 does not calculate Nei's (1973) diversity statistics directly, the components of Nei's diversity statistics can be obtained from other BIOSYS-1 subprograms. For example, Wright's total limiting variance (Wright, 1984) is equivalent to Nei's Ht statistic (Swofford and Selander, 1989), and the Fst statistic from Nei's F-statistics (Nei, 1977) is equivalent to the Gst statistic of Nei's diversity statistics (Swofford and Selander, 1989). Therefore, the above relationships (i.e., Ht = Hs + Dst and Gst = Dst/Ht) were used to calculate Hs and Dst of Nei's diversity statistics. All loci

were included in these calculations.

BIOSYS-1 was also used to calculate several similarity and distance coefficients. These included Nei's (1978) Unbiased Genetic Identity, Nei's (1978) Unbiased Genetic Distance, Roger's (1972) Genetic Distance, Prevosti Distance (Wright, 1984), Cavalli-Sforza and Edwards (1967) Arc Distance, and Edwards (1971, 1974) Distance. Each of these coefficients was further used in cluster analysis with unweighted pair group method analysis (UPGMA), weighted pair group method analysis (WPGMA), single linkage, and complete linkage.

A principal components analysis was also employed using the genetic data in order to further compare the relationship among pure, sympatric, and hybrid populations. A variance-covariance matrix was created, from which eigen vectors were calculated. Subsequently, the first three axes were extracted and the populations were plotted.

Contingency X^2 analysis was performed with BIOSYS-1 (Swofford and Selander, 1989) for each locus in order to estimate the extent of allele frequency heterogeneity among sympatric populations. This test was administered separately on each set of sympatric populations (Y/Z, AA/BB, and CC/DD).

Estimates of gene flow were calculated for the entire set of populations based on Wright's (1951) use of F_{ST} (and Nei's equivalent, G_{ST}). His equation, $Nm = \frac{1}{4}(1/F_{ST} - 1)$, is based on an island model of migration where every population is equally accessible to every other population. Crow and Aoki (1984) applied a correction factor of α to this equation to account for smaller sample sizes than those considered by Wright (1951). Alpha is calculated as $(n/n-1)^2$, where n is the number of populations being considered. With the application of α and substitution of G_{ST} for F_{ST}, Wright's equation becomes $Nm=$

$(1/G_{ST} - 1)/4\alpha$ (Crow and Aoki, 1984).

Comparison of Morphological, Geographical, and Genetic Distances

Mantel's test (1967) was used to determine if a relationship exists between

morphological, geographical distance, and genetic distance matrices. This is a

nonparametric test which analyzes two dissimilarity matrices in addressing the null

hypothesis that there is no association between the elements of one matrix and the

elements of another independently obtained matrix (Sokal and Rohlf, 1995). A Mantel

test statistic, Z, is computed as $Z= \sum X_{ij} Y_{ij}$, where X_{ij} and Y_{ij} are the off-diagonal

elements of matrices X and Y, respectively (Rohlf, 1988). Theoretically, if larger

distances in the X matrix match larger distances in the Y matrix, then Z will be larger than

expected by chance alone. Alternatively, if a negative association exists, (i.e., large values

of one matrix correspond to small values of the other matrix) then Z will be smaller than

expected by chance (Rohlf, 1988; Sokal and Rohlf, 1995). Because the Z statistic is

expressed in arbitrary units which are difficult to understand without tests of significance,

Smouse et al. (1986) have demonstrated that the ordinary product-moment correlation

coefficient, r, is directly related to Z, and is much easier to interpret. This Z statistic is

called the normalized Mantel statistic, and its significance is tested by comparing it to a

distribution of Z values created by random permutations of the elements of one matrix

while the elements of the other matrix remain fixed. Using NTSYS-pc (Rohlf, 1988), a

matrix of Average Taxonomic Distance between all pairs of populations was calculated for

the morphological characters and a matrix of Nei's (1978) Unbiased Genetic Distance

between all pairs of populations was calculated for the allele frequencies. A matrix of

geographic distances was created using direct air miles between all pairs of populations.

Because morphological measurements are only available for 27 of the populations, only these populations were used in all matrix comparisons. These distance matrixes were then compared to one another, in three distinct analyses corresponding to the distance matrix comparisons of morphology x isozymes, morphology x geography, and isozymes x geography. A matrix correlation coefficient, r, which is equivalent to the normalized Mantel Statistic, Z, is reported for each of the comparisons. The probability of the normalized Z statistic computed from 1000 random permutations being greater than or equal to the normalized Z statistic computed from the original comparison of matrices was calculated. A non-random association of the two matrices was inferred at $p \leq 0.05$.

Integration of Published Isozyme Data

In an effort to extend the work of Case (1993), the populations examined in this study were combined with populations of *C. parviflorum* vars. *makasin* and *pubescens* examined in her analyses. This increased the data set to 24 populations of var. *pubescens* and seven populations of var. *makasin* [all of the populations classified by Case as var. *parviflorum* are now assumed to be var. *makasin* based upon Sheviak's (1994) criteria; Table 2]. Additionally, five populations designated as mixed by Case (1993) are included. In order to include Case's data set in this research, it was necessary to know which alleles identified by Case correspond to those identified in this research. This alignment was made possible by re-collecting and assaying two highly variable populations that Case also examined. These populations are CC and DD in this research but are labeled P and Q, respectively, in Case (1993). The high frequency alleles identified in populations CC and DD by Wallace were assumed to be the same high frequency alleles discovered by Case. Because the vast majority of alleles within a locus in populations CC and DD have large

mobility and frequency differences, this inference was made with a high degree of certainty. However, some rare alleles were unable to be matched indisputably and were eliminated from both data sets. A total of nine alleles were excluded from the present populations while seven alleles were eliminated from Case's (1993) populations. The most common alleles found by Case (1993) are also the most common alleles found in this study. Two loci, PGI and GDH, were entirely eliminated because these loci were not included in Case's (1993) study. Lastly, Case's populations P and Q were not included in the statistical analyses of the combined data sets to avoid overrepresentation of these populations.

The combined data set was subjected to UPGMA using Nei's (1978) Unbiased Genetic Identities to further address the degree of relatedness among populations of vars. *pubescens, parviflorum,* and *makasin*. Additionally, Nei's (1973, 1977) diversity statistics were calculated over the range of populations such that these values could be compared to values obtained independently by Case (1993) and by myself in the present study.

In order to evaluate differences in the levels of genetic variation among northern populations (i.e., northern *pubescens* and *makasin)* and southern populations (i.e., southern *pubescens* and *parviflorum*) several diversity measures were calculated at the population level. To increase the size of the data set, Case's (1993) *pubescens* and *makasin* populations were included. Unlike UPGMA, there was no need to collapse or eliminate any alleles from loci common to both studies. However, two loci, PGI and GDH, were eliminated from all of my populations because they were not included in any of Case's (1993) populations. Likewise, MDH-3 was eliminated from Case's populations

because it was not included in my analyses. An artificial boundary was drawn to distinguish northern populations from southern populations of *pubescens* as well as *makasin* from *parviflorum*. All *pubescens* populations from Indiana and Ohio northward were classified as northern, and *pubescens* populations south of this were classified as southern (Table 1). Similarly, the geographic boundary defined by Sheviak (1994) was used to classify *parviflorum* and *makasin*. All of the *makasin* populations were from Michigan while *parviflorum* included all of the populations sampled in the current study from Indiana southward. Lastly, only populations containing a single variety (i.e., pure) were included in these analyses.

The average population size (i.e., number of genets), alleles per locus, percent polymorphic loci, and average expected heterozygosity were calculated for each population. A normal distribution of variates and equality of variances for A, P, and Hs permitted the use of a T-test (Sokal and Rohlf, 1995) to test for significant differences between northern and southern *pubescens* for each of these characters. Because population size was not normally distributed, a nonparametric Kruskal-Wallis test (Sokal and Rohlf, 1995) was used to test for significant differences in this character. Similarly, *parviflorum* and *makasin* were tested for significant differences among A and Hs via a T-test and population size and P via a nonparametric Kruskal-Wallis test.

In addition to testing for statistically significant differences between the groups, the relationship between variables within each group were also evaluated. Within each group (i.e., *pubescens* populations and *parviflorum/ makasin* populations) Spearman's rank correlation (SPSS, 1995) was calculated separately between population size and each of the following variables: number of alleles per locus, percent polymorphic loci, and

expected heterozygosity. Subsequently, significance levels were also calculated for each correlation.

Lastly, the amount of variation distributed among populations (Gst) was re-evaluated for northern *pubescens*, southern *pubescens*, *parviflorum*, and *makasin* populations with the inclusion of Case's (1993) populations. Alleles were collapsed just as they were in the UPGMA discussed earlier. Subsequently, a Gst value was calculated for each group based on these allele frequencies. Due to the absence of two loci, PGI and GDH, from Case's (1993) data and MDH-3 from my data, these loci were also eliminated from the calculation of Gst values.

RESULTS

Morphological Analysis

A histogram of estimated population size was produced to evaluate the typical size of *Cypripedium parviflorum* populations throughout the area sampled in this study (Fig. 2). Due to the occurrence of asexual reproduction via rhizomes in these plants, the number of unique genotypes or genets was also estimated by assuming each clump to be a single genet (Fig. 2). While the average population size of ramets is 132.4, the average number of unique genotypes per population is only 95.9 genets. However, these values should be interpreted with caution as the majority of populations consist of fewer than 100 individuals and fewer than 20 genets. Two populations have greater than 1000 plants which resulted in a considerable inflation of the mean.

Tests of significance among all groups for morphological characters revealed significant differences for all characters except the number of twists per lateral petal (Table 3). Pairwise tests of significance indicate that vars. *parviflorum* and *pubescens* are not significantly different in all of the vegetative traits (i.e., height, leaf length, leaf width) and one fertile trait, dorsal sepal length. However, variety *makasin* is significantly different ($p < 0.005$) from both var. *parviflorum* and var. *pubescens* in each of these characters. Variety *makasin* does not differ significantly from var. *pubescens* in only one trait, orifice length, while it is similar to var. *parviflorum* in five traits (staminode length, lateral sepal width, slipper length, slipper width, and orifice width). Three traits were found to be

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highly significantly different (p ≤ 0.001) for all pairwise comparisons between varieties.

These include petal length, dorsal sepal width, and lateral sepal length. Generally, there is

great overlap in the range of measurements for all varieties. Although petal width was

found to be significantly different in the Kruskal-Wallis test, intervarietal significance

could not be tested due to the high number of tied ranks (52) of one value. Although

Dunn (1964) says that the presence of many ties should not affect the test statistic, I found

that the standard error could not be computed for comparisons between

parviflorum/makasin (N=50) or between *pubescens/makasin* (N=49).

Principal components analysis of vegetative characters did not separate individuals

into distinguishable clusters (Fig. 3). PCA performed on flower characters did, however,

reveal apparent groups among the varieties. Individuals labeled as *parviflorum* and

makasin largely grouped together on the left side of the plot while those individuals

labeled as *pubescens* usually grouped together on the right side (Fig. 4). With the vast

amount of variation present in this species, it is no surprise that some individuals

representing all three varieties overlap in the central portion of the plot. The first three

axes explain 78% of the variation, and are most strongly correlated with variation in petal

length, dorsal sepal dimensions, and slipper length (1st axis); the number of twists/lateral

petal (2nd axis); and orifice length (3rd axis; Table 4).

A principal components analysis of mean population character values for all

measured characters produced results similar to the analysis of floral character values for

individuals. Most *parviflorum* and *makasin* populations cluster together and most

pubescens populations cluster together (Fig. 5). The one population classified as having

individuals of two varieties as well as intermediate morphologies, clusters closer to pure

pubescens populations than to pure *parviflorum* populations. The first axis explains 64%

of the variation and is most strongly correlated with petal length, dorsal and lateral sepal

dimensions, and slipper length (Table 5). The second and third axes account for another

19% of the variation.

The Average Taxonomic Distance was calculated between all pairs of populations

(Appendix 2). Population X contained individuals of variety *parviflorum*, variety

pubescens, as well as hybrid morphologies. Therefore, it was not included in calculations

of intravarietal or intervarietal distance. Intervarietal comparisons indicate that

parviflorum and *makasin* populations are the least distant ($\overline{D}$=1.103) and *makasin* and

pubescens populations are the most distant ($\overline{D}$=1.703; Table 6). Within each of the

varieties, mean population distances are 1.029, 1.026, and 0.616 for *pubescens*,

parviflorum, and *makasin*, respectively.

Cluster analysis was also performed using population mean values for all

characters. Average Taxonomic Distance, Euclidean Distance, and Manhattan Distance

with UPGMA clustering produced similar phenograms with similar cophenetic

correlations. The combination of Average Taxonomic Distance with UPGMA resulted in

the highest cophenetic correlation (73%). Therefore, only the results of this analysis will

be reported herein. The Average Taxonomic Distance between populations in conjunction

with UPGMA produced the phenogram in Figure 6. Similar to PCA, the cluster analysis

also produced evident clusters of each of the varieties. The phenogram depicts two main

clusters defined as a *pubescens*-like branch and a *parviflorum-makasin* branch. Although

the *parviflorum* populations P, Q, and U appear to have morphologies similar to

pubescens populations, they represent a distinct sub-branch within the *pubescens* cluster.

Based on the morphological characters used to produce the phenogram, *makasin*

populations cluster together but do not appear to be systematically distinct from

parviflorum populations.

Isozyme Analysis

Of the 18 putative loci resolved on ten staining systems, 13 are included in these

analyses. Three loci were observed for GOT and MDH. GOT-3 was consistently

unresolvable while MDH-1, MDH-2, and MDH-3 showed highly variable banding patterns

which could not be interpreted genetically based upon all known reports of its quaternary

structure. These loci, therefore, were omitted from all analyses. PGM, TPI, and IDH

each had two loci. However, IDH-1 was unresolvable and was excluded from analysis.

The remaining isozymes, PGI, ADH, GDH, SOD, SKD, and PGD, each exhibited one

locus. All individuals were scored for the 13 loci that were consistently resolvable.

Two loci, GOT-1 and SOD, were found to be monomorphic in all populations. All

other loci were polymorphic. One null allele was found for GOT-2. It's presence in a

heterozygous state was consistently detectable, and therefore is included. Allele

frequencies are given for each population (Appendix 3) and each variety (Table 7). At the

species level, *C. parviflorum* is polymorphic at 85% of all loci (Table 8). Similarly, for

any given variety 85% of loci are polymorphic. All varieties were polymorphic for the

same suite of loci. At the population level, 53% of the loci are polymorphic ignoring

varietal boundaries, while populations within vars. *parviflorum*, *pubescens*, and *makasin*

have average population polymorphism at 42%, 56%, and 77% of their loci, respectively.

The average number of alleles per locus was found to be 3.23 for the species. For

any given variety, A_v is lower, ranging from 2.38 in var. *parviflorum* to 2.77 in var.

pubescens (Table 8). Based on the total number of alleles present in one variety or the other, the varieties share 28 (70%), 29 (73%), and 28 (80%) of these alleles, respectively, between *pubescens* and *parviflorum*, *pubescens* and *makasin*, and *parviflorum* and *makasin* (Table 9). Nine unique alleles were found across the three varieties with *parviflorum* and *makasin* each having two while *pubescens* has five (Table 9) . Five of these alleles are considered private alleles (Slatkin, 1985; Barton and Slatkin, 1986) as they occur in only one population. These include GDH-1f, PGM-1a, PGD-1a, PGI-1c, and ADH-1a. Of the private alleles, *pubescens* contained four, *parviflorum* one, and *makasin* none. The average frequency of alleles unique to a single variety is 0.025 while that of private alleles is 0.004.

Observed versus expected heterozygosity estimates averaged across all loci for each population are given in Table 8. Observed heterozygosity ranged from 0.068 in population C to 0.308 in population I, and expected heterozygosity ranged from 0.060 in population AA to 0.258 in population V. The average of 0.174 for observed heterozygosity over all loci and all populations is extremely close to the expected average of 0.175.

Out of 206 single locus tests across 30 populations, 17 loci (8.25%) were found to have genotype frequencies that differed significantly ($p \leq 0.05$) from Hardy-Weinberg equilibrium expectations. Twelve populations had at least one locus out of equilibrium, and four populations had more than two loci out of equilibrium (Table 10). Among the latter, populations B, J, and V have two loci and population C has three loci with significant deviations. Each of these populations exhibits a deficiency of heterozygotes. Furthermore, the fixation index (1 - observed heterozygosity/expected heterozygosity), a

measure of the reduction in the number of heterozygous individuals within a population

(Wright, 1965; 1984), is 1.0 for each of the loci out of equilibrium in population C.

The total amount of diversity as measured by species level expected heterozygosity

across all loci ranges from 0.182 in var. *parviflorum* to 0.280 in var. *makasin* (Table 11).

With the inclusion of all populations, the species level diversity is 0.213. There is a

relatively smaller amount of variation that is distributed among populations of each

variety. Populations of var. *makasin* partition only 11% of their variation among

populations while populations of vars. *parviflorum* and *pubescens* are slightly more

differentiated with 19% and 20% of the variation partitioned among populations,

respectively. See also Appendix 4 for single locus diversity statistics of each taxon and

standard errors of loci. A hierarchical analysis of the species diversity based on the

method of Wright (1984) revealed that 82% of the variation is contained within

populations, 15% is among populations within varieties, and only 3% is among varieties.

Nei's (1978) Unbiased Genetic Identity was calculated between all pairs of

populations (Appendix 2). Because population X included hybrid morphologies of vars.

parviflorum and *pubescens*, it was excluded from calculations of mean intravarietal and

intervarietal identity values for both of these varieties. Intravarietal measures of

population identity yielded high mean values of 0.965, 0.965, and 0.943, respectively for

vars. *pubescens*, *parviflorum*, and *makasin* (Table 6). Intervarietal comparisons show

parviflorum and *pubescens* populations to be the most closely related with a mean genetic

identity of 0.963. The comparison of *pubescens/makasin* and *parviflorum/makasin*

yielded very similar mean identities of 0.901 and 0.902, respectively.

The use of Nei's (1978) Unbiased Genetic Identity in conjunction with UPGMA produced a phenogram with the lowest percent standard deviation (2.341; Fitch and Margoliash, 1967) of all possible combinations of similarity/distance coefficients and clustering algorithms. Furthermore, the cophenetic correlation for this method was 0.82. Thus, only the results of this analysis will be reported herein. Although no clustering of populations of either variety *parviflorum* or *pubescens* is apparent from the UPGMA (Fig. 7), all populations of these varieties are grouped at a high similarity of approximately 0.92. In sharp contrast, the populations of variety *makasin* clustered together and joined the remainder of populations at a similarity of approximately 0.90. Two of the pairs of sympatric populations (AA/BB, CC/DD) did not cluster near one another. Surprisingly, geographically close populations of the same variety did not cluster together either (e.g. POPS B/C, D/E/F, H/I).

A principal components analysis of allele frequencies produced a similar distribution of population clustering to that from UPGMA. All of the *makasin* populations (V, W, CC) are delimited from the large clump of *parviflorum* and *pubescens* populations (Fig. 8). Notably, however, *parviflorum* population T appears more closely positioned to the *makasin* populations than to any other *parviflorum* population. The PCA indicates that var. *parviflorum* is virtually indistinguishable from var. *pubescens* based on allele frequencies. The first principal component axis is most strongly correlated with variation at TPI-2, GDH-1c, and IDH-1a,b (Table 12). The first three axes together account for 61% of the variation seen among the populations.

To explore the extent to which introgression may have occurred in sympatric populations, X^2 contingency analyses were performed on the three pairs of sympatric

populations (POPS Y/Z, AA/BB, CC/DD). Within each pair of populations highly

significant (p < 0.001) overall levels of allele frequency heterogeneity exist (Table 13). In

comparisons between populations Y and Z, five of 10 polymorphic loci show significant

allele frequency differences. The analysis of populations AA and BB show only one out of

seven polymorphic loci to have nonsignificant differences in allele frequencies, and

populations of CC and DD have seven of 10 polymorphic loci that are significantly

different.

Estimates of the amount of gene flow occurring between populations for the

species are 0.809 migrants per generation based upon a G_{ST} value of 0.224. Similar

estimates were observed for each of the varieties. They ranged from 0.831 migrants per

generation in *parviflorum* to 0.908 migrants per generation in *pubescens*.

Comparison of Morphological, Geographical, and Genetic Distances

The Mantel (1967) test for matrix association resulted in nonsignificant

correlations between the distance matrix comparisons of morphology x allele frequencies

(r = 0.136, p > 0.05; Table 14) and for morphology x geographic distance (r = 0.003, p >

0.05; Table 14). Lastly, there is a low but significant correlation between the geographic

distance and genetic distance matrices (r = 0.162, p < 0.01; Table 14). This indicates a

slight positive relationship between genetic distance and geographic distance.

Integration of Published Isozyme Data

The analysis of Case's (1993) data with data from the current study produced

average genetic identity values ranging from 0.914 in var. *makasin* to 0.979 in var.

parviflorum. The average genetic identity for intravarietal comparisons is 0.954 for the

integrated data set (Table 6). Varieties *pubescens* and *parviflorum* have similar values in

the integrated data set ($\bar{I}$ = 0.974) and in the current study ($\bar{I}$ = 0.963). The intervarietal

comparisons of *pubescens/makasin* ($\bar{I}$ = 0.898) and *parviflorum/makasin* ($\bar{I}$ = 0.892)

also show very similar values to those reported in the current study ($\bar{I}$ = 0.901, $\bar{I}$ = 0.902,

respectively; Table 6).

In UPGMA using both data sets, no single variety clustered entirely away from any

other variety. Populations from the present study are mixed with populations from Case's

(1993) study throughout the phenogram (Fig. 9). Several branches of *pubescens*

populations can be observed, and notably, the *makasin* populations cluster on separate

branches from *parviflorum* populations. All populations cluster at an average identity of

0.80, considerably lower than the identity of 0.92 observed in the analysis of the smaller

set of populations from the present study.

The inclusion of Case's (1993) populations with the populations in the current

study resulted in a slightly higher estimate of genetic diversity than reported for my

populations (Ht = 0.215; Table 11). However, the partitioning of genetic variation among

populations is similar in all three data sets [i.e., 23% of the variation resides among

populations in the larger set compared to 22% for my populations and 19% reported by

Case (1993)].

Although the species level estimate of among population variation (Gst) is similar

when either populations in this study or the larger set of populations [i.e., including Case's

(1993) populations] are considered, the variety estimates are remarkably different.

Makasin populations exhibit higher amounts of among population variation (27%; Table

11) with the inclusion of Case's populations than when only populations of the present

study are evaluated (11%), or only Case's (1993) populations are considered (17%). The

analysis of southern *pubescens* populations indicates that 23% of the variation is maintained among populations. This is compared to 16% for northern *pubescens* populations and 20% for all *pubescens* populations included in the present study. A comparison of northern and southern *pubescens* populations is given in Table 15. The mean population size of northern *pubescens* is substantially greater than the average population size of southern *pubescens*. Likewise, the other measures of genetic diversity (i.e., A, P, and Hs) are also higher for northern *pubescens*. A T-test (Sokal and Rohlf, 1995) determined the differences to be significant for each of these variables between the two groups (Table 15). The disparity in measures of genetic diversity is even greater between *parviflorum* and *makasin*. For example, *makasin* populations have on average 73% polymorphic loci while *parviflorum* populations maintain only 37% polymorphic loci (Table 16). Furthermore, the average population size of *makasin* populations is 13 times greater than the average size of *parviflorum* populations. All variables demonstrated significant differences between *parviflorum* and *makasin*.

The correlation analysis provided some interesting results. Within northern and southern *pubescens* populations, the number of alleles per locus and percent polymorphic loci are significantly associated ($p < 0.05$) with genet size (Table 17). However, average expected heterozygosity is not significantly correlated with genet size. In *parviflorum* and *makasin* populations only alleles per locus was found to be significantly correlated with genet size (Table 17).

DISCUSSION

Morphological Variation

The extensive morphological variability and overlap in character ranges among putative infraspecific taxa of *C. parviflorum* have been recognized for decades (e.g., see Correll, 1938). According to Sheviak (1983), this species has "generated probably more thought and contradictory pages of print than any other North American orchid." Results from the univariate and multivariate statistics conducted in this research demonstrate that there are no discrete, non-overlapping quantitative characters in the data set that can reliably be used to classify an individual into any given variety. This finding is consistent with previous analyses that have examined infraspecific taxa of *C. parviflorum* for the existence of discriminating characters. For example, Newhouse (1976) found that 11 quantitative and eight qualitative characters differed significantly between var. *parviflorum* (= var. *makasin* as described in this study) and var. *pubescens*. Furthermore, 11 of these variables (quantitative and qualitative) also exhibited overlapping ranges between varieties. Of the eight non-overlapping variables, six were qualitative characters including fragrance, color of the lateral petals, color of markings inside the slipper, stem pubescence, leaf pubescence, and flower pubescence. Only two quantitative traits, lip height and number of twists per lateral petal, did not have overlapping ranges between *pubescens* and *makasin*. For variety *makasin*, the mean values obtained for individuals

sampled in this study were comparable to the means reported by Newhouse (1976) in all

characters except the number of twists per lateral petal. For this character, Newhouse

(1976) found an average of approximately 5.3 twists per petal and a range of 4.0 to 7.0

for var. *makasin*. Variety *pubescens*, she found, had significantly fewer twists ($\overline{X} = 2.7$)

and an approximate range of 1.0 to 4.0. In my data, there were no significant differences

among any groups for this character.

Generally, the mean value as well as the lower and upper range limits I found for

var. *pubescens* are greater than the estimates obtained by Newhouse (1976). Dorsal sepal

width was the only quantitative character measured by Newhouse (1976) which exhibited

a wider range than the range I observed in var. *pubescens*. I found a substantially higher

mean than reported by Newhouse (1976) for three characters (dorsal sepal length, the

number of twists per lateral petal, and petal length). Although the lower range limits for

each of these characters are similar between the two studies, the upper limits I found are

much higher than Newhouse's (1976) values. The mean and lower and upper range limits

of the other quantitative characters are similar between the two studies. The fact that the

range limits increase with the inclusion of populations from the south may reflect a greater

geographical partitioning of morphological variability in var. *pubescens* than either of the

other varieties.

Even though individuals cannot be assigned unequivocally to a given variety,

significant differences in mean rank scores (Kruskal-Walllis test) were found among

various combinations of varieties for the 14 quantitative characters measured. Of these

characters, 13 were found to be significantly different among at least one pair of taxa.

Varieties *makasin* and *parviflorum* are not significantly different from each other in

staminode length, lateral sepal width, slipper dimensions, or orifice width (Table 3). Each of these characters, however, differs significantly from *pubescens*. Generally, floral characters were most similar among *parviflorum* and *makasin* whereas vegetative characters were most similar among *parviflorum* and *pubescens*. The latter two varieties did not differ significantly in height, leaf length, leaf width, or dorsal sepal length. However, each of these characters was significantly different from *makasin*. Three characters (petal length, dorsal sepal width, and lateral sepal length) were significantly different among all three varieties. The significant morphological differences found in 13 out of 14 quantitative characters measured in this study suggest that three statistical groups exist (*pubescens, parviflorum,* and *makasin*), but not all characters are consistent in delimiting the three groups.

Consistent with what is most commonly reported in the literature (e.g., Sheviak, 1995; Newhouse, 1976; Correll, 1938), *pubescens* appears to be the most robust taxon with generally larger features than *parviflorum* and *makasin*. In addition, it can display relatively large amounts of morphological variability. Individuals are known to vary morphologically from year to year, and may change dramatically when transplanted to a different habitat (Sheviak, 1995). Some authors have even insisted that *parviflorum* can change into *pubescens* upon transplantation to a more suitable habitat (in Sheviak, 1995). However, it is more likely that a diminutive *pubescens* becomes more robust when transplanted (Sheviak, 1995). In seven of the 14 characters measured, *pubescens* exhibited the greatest range in character values. Variety *parviflorum* also displayed character ranges similar to *pubescens* but slightly exceeded the ranges of *pubescens* for two characters, height and leaf width (Table 3). In contrast, the character ranges of var.

makasin were very small compared to the ranges for either *pubescens* or *parviflorum*. For most characters, the ranges for *makasin* were about one third as large as the ranges for *parviflorum*. This can also be seen in the intravarietal taxonomic distances. Populations of var. *makasin* have an Average Taxonomic Distance of 0.616 whereas *pubescens* and *parviflorum* have intravarietal taxonomic distances of 1.029 and 1.026, respectively (Table 6). This indicates a high degree of similarity among the *makasin* populations sampled. Although this result suggests that there may be less variation in the characters for var. *makasin*, it could also reflect a relatively small sample size of individuals for this taxon (N=3 populations).

Of the eight quantitative characters that are common to both Newhouse's (1976) study and the present study, five were found to be significantly different between *pubescens* and *makasin* in both studies. These include slipper length, slipper width, lateral petal length, dorsal sepal length, and dorsal sepal width. Although Newhouse (1976) reports significant differences in the number of twists per lateral petal and lateral petal width between *pubescens* and *makasin*, I did not find a significant difference in the number of twists per lateral petal between *pubescens* and *makasin* (Table 3). In contrast to the nonsignificant results reported by Newhouse (1976), vars. *pubescens* and *makasin* do differ significantly in height for the populations sampled in this study. An interesting result of Newhouse's (1976) study is that the degree of shade and soil moisture, factors which likely contribute to plant height, differed significantly between the two varieties. The discrepancy in significance of plant height between Newhouse's (1976) and the current study may reflect greater morphological uniformity among *parviflorum* and *pubescens* in vegetative traits for southern populations. The majority of *pubescens* populations

included in the present study are from the southeastern United States whereas all of

Newhouse's (1976) *pubescens* populations were located in Michigan. Sheviak (1995) has

noted that populations of *pubescens* from eastern deciduous forests do not display the

morphological variability seen in *pubescens* populations from other areas. This may also

reflect the greater habitat uniformity in the southeastern forested landscape. The data

presented here would seem to indicate that the southern habitat (i.e., shady areas) is

conducive to producing tall plants with large spreading leaves in both var. *pubescens* and

var. *parviflorum*. The greater degree of morphological variability in floral characters

among *parviflorum* and *pubescens* in the south relative to vegetative characters suggests

that floral traits are either not as affected by environmental conditions or have evolved

differences independently from the stems and leaves.

In general, PCA and UPGMA also indicate that the data contain some

recognizable groups, although considerable overlap exists among individuals of the

groups. The most well defined groups are *pubescens* and *parviflorum/makasin*. Most

pubescens individuals cluster together and away from *parviflorum* and *makasin*. For

example, the largest taxonomic distance in the UPGMA separates all *pubescens* from

makasin and most of *parviflorum* (Fig. 6). The three *makasin* populations cluster

together on the UPGMA but are within a larger *parviflorum* cluster. Likewise, on the

PCA, *makasin* populations cluster in a similar region but are dispersed throughout

parviflorum.

Three *parviflorum* populations (P,Q,U) cluster well away from the other

parviflorum on the PCA and UPGMA. These populations had the coloration of the sepals

and petals and slipper size of *parviflorum*, but were otherwise more like *pubescens* in one

or more characters. For example, in population P some individuals exhibited petals ranging from 4.25 to 7.25 cm in length. The upper range exceeds the mean petal length for var. *pubescens* ($\overline{X} = 7.1$; Table 3). Individuals in population P also have lateral sepal lengths more similar to *pubescens* than to *parviflorum*. Lastly, these plants were taller than many *pubescens* individuals sampled. Similar to population P, individuals of population Q were tall and had long petals and lateral sepals. Population U also contained robust plants with the color characteristics of *parviflorum*. However, unlike the previous two populations, U had petals and lateral sepal lengths which were more similar to the averages for var. *parviflorum*. In summary, each of these three populations exhibited color characteristics and slipper dimensions of var. *parviflorum*. However, other traits (e.g., plant size, petal length, and lateral sepal length) appear to be robust, a characteristic most commonly found in var. *pubescens*. The robustness in quantitative characters probably accounts for the apparent clustering of these *parviflorum* populations with *pubescens* populations in both PCA and UPGMA. The inclusion of qualitative characters such as color may be necessary to produce discrete groupings of *parviflorum* and *pubescens* populations.

Sheviak (1994) has largely used qualitative characters such as density and color of pubescence, fragrance, geographic distribution, and overcoloring of the sepals and petals to classify the varieties. However, these traits may also be problematic because pubescence may exist in degrees, fragrance may be variable (e.g., Wallace, pers. obs.; Newhouse, 1976), discrete geographical boundaries are debatable (e.g., Luer, 1975; Fernald, 1946, 1950; Correll, 1950), and petal color is subject to variation (e.g., Sheviak, 1994, 1995; Atwood, 1985). Most notably, Sheviak (1994) argues that var. *makasin* has

a sheathing bract near the base of the stem which is sparsely pubescent to glabrous when young. In contrast, vars. *parviflorum* and *pubescens* exhibit a similarity in pubescence which consists of densely arranged silvery hairs on young plants. However, with age, the sheathing bract of vars. *parviflorum* and *pubescens* may also become glabrous (Sheviak, 1994). Although Sheviak maintains that this is a characteristic easily seen on live or pressed specimens, I was unable to detect a difference among any of the varieties. However, Sheviak does not mention how old plants must be when this pubescence disappears. It is possible that the plants I surveyed were old enough to have lost their pubescence. Newhouse (1976) found significant differences in the amount of pubescence on stems, leaves, and flowers between vars. *pubescens* and *makasin*. On all three areas, *pubescens* was more pubescent than *makasin*. Another area in which the three varieties differ is scent. Newhouse's (1976) data support this distinction as she found var. *makasin* to have a strong scent and *pubescens* a weak scent. Sheviak (1994) also describes var. *makasin* to have a strong fragrance while vars. *pubescens* and *parviflorum*, he maintains, have a lighter scent which may be rose or "pungent-musty". I initially could detect both sweet and musty scents variably in both *pubescens* and *parviflorum*. Bergstrom et al. (1992) did find that vars. *parviflorum* and *pubescens* contain different fragrance compositions. However, it is not clear whether their interpretation of *parviflorum* represents *parviflorum* or *makasin* as I have interpreted them here.

Geographic distribution is a difficult character to quantify in *C. parviflorum* because there are no obvious changes in morphology that would clearly separate populations of var. *makasin* and var. *parviflorum* at the apparent geographical species boundaries. According to Sheviak (1994), var. *makasin* occurs in New England and

Canada west to the Canadian prairies and northern cordilleran, and var. *parviflorum* occupies a range from southern New England south to Georgia and westward across the lower Midwest. The morphological data presented in this research does not support this geographic distinction.

Both vars. *makasin* and *parviflorum* are characterized by dark reddish brown pigmentation on the sepals and petals. According to Sheviak (1994), *makasin* has a suffusion of color compared to *parviflorum* which exhibits a dense spotting of color provided by "individual spots arranged in closely spaced longitudinal rows". Contrary to this, I have observed plants well within the geographic range of *parviflorum* with sepals and petals that are entirely pigmented purplish black and are indistinguishable from *makasin* in this character. I have also seen plants with very large slippers and dark pigmentation throughout the sepals and petals which resemble *pubescens* in quantitative characters and *parviflorum* in qualitative characters. Remarkably, the initial classification of var. *pubescens*, or vars. *parviflorum* and *makasin* populations in the field based upon color characters and slipper dimensions generally was supported by the clustering of convarietal populations in PCA and UPGMA based on the suite of quantitative characters measured. It appears that a combination of comparative quantitative and qualitative characters may be most effective in the delimitation of these taxa in the field.

In conclusion, results from the morphological analyses indicate that *parviflorum* and *pubescens* form the most well defined groups, and that *makasin* is very similar to, if not indistinguishable from *parviflorum*. The Average Taxonomic Distance supports this as the lowest distance is between *parviflorum* and *makasin*. This value is very near the intravarietal values reported for both *pubescens* and *parviflorum* (Table 6). Lastly, no

qualitative characters were observed in the field (or from subsequent examination of voucher specimens) that would delimit vars. *makasin* and *parviflorum*.

Isozyme Variation

Intervarietal Patterns

Unlike the morphological data which indicates that the most recognizable groups are *parviflorum* and *pubescens*, these two taxa do not form recognizable clusters in the UPGMA of genetic identity values (Fig. 7). Mean genetic identity of *parviflorum* and *pubescens* comparisons (0.963) is very close to mean intravarietal comparisons of *pubescens* 0.965 and *parviflorum* (0.965). Furthermore, some of the intravarietal comparisons of *pubescens* (e.g., 0.825) were lower than the lowest intervarietal comparison of *pubescens* and *parviflorum* (e.g., 0.881). Similar results are obtained using the combined data set of populations collected for this study and populations from Case (1993) (Table 6, Fig. 9). Therefore, *parviflorum* and *pubescens* are indistinguishable based on isozyme data.

Populations containing *makasin* (in either pure or mixed populations) form the most recognizable group. This is most evident in the analysis that used populations from Case (1993) combined with those in the present study. In Figure 9, only two (SS and TT) of the 12 populations containing *makasin* individuals cluster out of the predominantly *makasin* branch near the bottom of the phenogram.

Although *makasin*-containing populations cluster together, there is considerable variance in the degree of genetic identity among the populations. For example, in the data set that combines the populations from Case (1993) with those of the present study, *makasin* intravarietal values have the largest range in values (0.787-0.991; Table 6).

Likewise, the lowest intervarietal comparisons occurred when *makasin* was compared

with either *pubescens* or *parviflorum* (0.635 and 0.748, respectively; Table 6). These

results suggest that *makasin* populations can be as dissimilar to each other as they are to

pubescens or *parviflorum* populations. Therefore, it is difficult to define *makasin*

populations based on allele frequencies. It should be noted that *makasin* populations also

contain the largest amount of among-population genetic variance as well as the largest

amount of genetic variation within populations (see discussion below).

Principal components analysis shows that *parviflorum* and *pubescens* populations

are dispersed throughout a similar region (Fig. 8), and do not resolve into separate

clusters. This is qualitatively consistent with the results from UPGMA. Although

makasin populations cluster in a common region on the PCA plot, there is a large amount

of variance among them on axis two. Furthermore, the three axes together only explain

60% of the total variation with relatively high amounts of variance dispersed among the

three plotted axes. These results confirm that the various allele frequencies are relatively

uncorrelated with each other. Consequently, no highly resolved groups can be found in

the data.

The varieties are also difficult to define on the basis of unique alleles. For

example, only five unique alleles were found in var. *pubescens*, but these alleles were

confined to two or fewer populations each. Likewise, the two unique alleles found in

vars. *parviflorum* and *makasin*, respectively, were also only found in two or fewer

populations each. Therefore, these unique alleles might be best thought of as population

specific rather than variety specific. Additionally, in 11 of the 13 loci surveyed, the

highest frequency allele in the species was the highest frequency allele at the varietal level

and usually the highest frequency allele in each of the populations. These results demonstrate that subtle allele frequency differences among the populations are responsible for the clustering patterns seen in UPGMA and PCA rather than the presence of any diagnostic alleles.

Overall, *C. parviflorum* exhibits high levels of genetic identity between populations ($\bar{I}$ = 0.922). This is consistent with Case's (1993) assessment of *C. parviflorum* in which she found a mean intervarietal identity of 0.92 and mean intravarietal identities ranging from 0.92 to 0.98. The value reported here is comparable to the average genetic identity for conspecific populations ($\bar{I}$ = 0.95; Gottlieb, 1977) as well as reports for other subspecific taxa (e.g., Crawford and Smith, 1984; Wolf et al., 1991; McLeod et al., 1983; Heywood and Levin, 1984). For example, Crawford and Smith (1984) analyzed the genetic variation in four morphologically variable varieties of *Coreopsis grandiflora* Hogg ex Sweet and found an average genetic identity of 0.91 for all populations surveyed. Intervarietal comparisons were equally high and ranged from 0.79 to 0.99. Similarly high intravarietal and intervarietal genetic identities have been observed in other taxa including *Gaillardia pulchella* Foug. and its associated varieties (Heywood and Levin, 1984) and the *Ipomopsis aggregata* (Pursh) V. Grant complex consisting of eight subspecies (Wolf et al., 1991). In contrast, several infraspecific taxa have been shown to be isozymically divergent as they have genetic identities which are much lower than the identity values commonly reported for such taxa. For example, two varieties of *Coreopsis cyclocarpa* Blake had a mean genetic identity of 0.75 compared to intravarietal identities of 0.95 and 0.98 (Crawford and Bayer, 1981). Likewise, Rieseberg et al. (1987) found much lower mean intervarietal identities ($\bar{I}$ = 0.84) than intravarietal identities ($\bar{I}$ = 0.93-0.98) for the

four varieties of *Allium douglasii* Hook. Infraspecific taxa which exhibit lower than expected genetic identities are believed to have diverged isozymically after the disruption of gene flow among taxa (Heywood and Levin, 1984). Because the varieties of *C. parviflorum* show a high degree of genetic similarity, it is expected that they have recently experienced gene flow and have not diverged completely yet. The moderately high level of gene flow (Nm=0.809) in the species has apparently caused a high genetic similarity among varieties or very recent phylogenetic divergence. This is also indicated by a very low percentage of total variation in the species that resides among the varieties (3%).

Intravarietal and Geographic Patterns

Populations of *C. parviflorum* exhibit characteristics typical of outbreeding, long-lived herbaceous perennials. Generally, populations of this species have relatively high levels of genetic diversity, a lack of deviation from Hardy-Weinberg equilibria, and moderate levels of among population variation. Percent polymorphic loci at the species and varietal levels was 84.6%, and mean population values were 41.8% (in *parviflorum*), 55.5% (in *pubescens*), and 76.9% (in *makasin*). In comparison, species with either a similar widespread distribution or herbaceous perennial habitat have on average 58.9% and 39.6% polymorphic loci, respectively (Hamrick and Godt, 1989). Additionally, the estimate in the present study is higher than that reported by Case (1993) for the species (P=75%). A similar trend is seen in the number of alleles per locus at the species level.

Expected heterozygosity levels for all populations in this study averaged 0.175 and also followed a varietal-trend similar to the trend for polymorphic loci. *Makasin* had the highest level of expected heterozygosity, followed by *pubescens*, then *parviflorum* (Table 8). With few exceptions, most loci in most populations conform to Hardy-Weinberg

expectations. Out of 206 total tests, only 17 (8.25%) exhibited significant deviations (Table 10). Therefore, it is likely that most populations outbreed as opposed to a regular mode of inbreeding. This is consistent with the pollination syndrome of the species which is thought to prevent autogamy (Van der Pijl and Dodson, 1966; Stoutamire, 1967; Newhouse, 1976). In addition, the floral biology may also prevent high levels of geitonogamy. Since pollinators are temporarily trapped inside the flower with no reward, the impetus to immediately pollinate a neighboring flower upon escape might be reduced. One population (population C) did display deviations consistent with inbreeding at three loci. Because this population contained only nine genets, it might be expected to display inbreeding patterns. However, most populations composed of small numbers of genets (e.g., populations D-F, I, and BB) did not display any deviations from Hardy-Weinberg equilibria that were consistent with inbreeding.

Within the varieties, populations typically displayed moderate levels of among population variation. Varietal Gst values for populations in this study were the lowest for *makasin* (0.114), but were similar in value for *parviflorum* (0.192) and *pubescens* (0.196). However, it is likely that the relatively low Gst for *makasin* is due to a small sample size of populations for this taxon in the present study (i.e., 3 populations). When *makasin* populations from Case (1993) are combined with populations from the current study, the Gst increases to 27% (Table 11).

In addition to the slight differences in the overall levels and distribution of genetic variation among the three varieties, differences in the levels of variation were also found geographically. Alleles per locus, polymorphic loci, and population heterozygosities were significantly lower in southern populations of var. *pubescens* than in northern populations

(Table 15). Likewise, *parviflorum* populations (which by definition are southern) held significantly lower levels of genetic variation than northern *makasin* populations (Table 16). In twenty southern populations analyzed, 15 populations included electrophoretic samples from every genet in the population. In the remaining five populations, approximately 40%-86% of the entire population was collected. Therefore, the differences in variation between the north and the south reflect true population variation differences rather than merely sampling effect differences. Further sampling in the north may have yielded an even greater disparity in levels of variation among the north and south.

These results suggest that the factors affecting northern vs. southern variation in *pubescens* may have also affected the variation levels in *makasin* and *parviflorum*. Glaciation events have been extremely important in shaping the evolutionary history of many plant species especially in temperate areas (e.g., Hoey and Parks, 1991; Hawley and DeHayes, 1994; Qiu and Parks, 1994). The last North American glaciation began approximately 100,000 years ago and retreated 10,000 years ago (Dawson, 1992). Based upon the genetic identity between taxa and mutation rate of 10^{-7}, Nei (1987) developed a formula for approximating the time of separation between taxa. From Table 9.2 in Nei (1987), the approximate time of separation between the varieties is 200,000 to 600,000 years ago. Given their potential time of separation, the last ice age may have had great impact on the colonization history of the varieties by affecting the genetic structure evident today among populations of the north and the south.

As glaciers retreated out of Michigan and adjacent states, it is likely that the early deglaciated land created highly suitable habitat for *C. parviflorum* populations. The

populations closest to the retreating glacial front may have been very large with a more contiguous distribution compared to more southern populations. The latter may have been more intermixed with woody flora and more patchy in their distribution. As a consequence, the northern populations may have maintained higher levels of interpopulation gene flow. These conditions would be more suitable for the maintenance of higher levels of genetic diversity than the smaller, more isolated populations which may have occurred in southern areas.

Geographic structuring of genetic variation is not unique to *C. parviflorum* populations. Other species including *Picea rubens* Sarg. (Hawley and DeHayes, 1994) and *Pseudotsuga menziesii* (Mirb.) Franco (Li and Adams, 1989) contain populations in unglaciated areas which exhibit much lower levels of genetic diversity than populations in once glaciated regions do. In studying genetic variation in red spruce, Hawley and DeHayes (1994) found a gradual increase in levels of genetic variability along a cline from the southernmost localities to the northernmost. After ruling out introgression with black spruce, they hypothesized that the lower levels of genetic diversity in southern populations compared to northern populations was a consequence of genetic drift and inbreeding. Data that suggested this include a high degree of genetic differentiation among southern populations, higher than expected levels of inbreeding in southern populations, and the possibility of reduced gene flow among populations. They proposed that northern and southern populations were derived from different glacial refugia which initially had unequal levels of diversity. Furthermore, past migration patterns, selection pressures, and climatic differences may have enhanced expansion of populations in the north at the same time reducing and isolating populations in the south.

Presently, there are noticeable differences in the availability of suitable habitats in Michigan compared to more southern states. In Michigan, especially along tracks of calcareous Lake Michigan shoreline, it is not uncommon for Lady's Slippers to be found continuously for several miles (Case, personal communication). In the southern states visited, this condition was never found (pers. obs.). Furthermore, the population sizes appear to be much larger in Michigan. In these data, there were significant differences in the sizes of the populations sampled. In the south, average size of the populations visited for this study was 18 genets. In the northern populations, average population size was 353 genets. It should be noted that while contacting botanists for locations, I sought the largest known populations from which to sample. Therefore, it is likely that the choice of populations examined reflects an actual difference in population sizes between the north and the south.

Among all northern and southern populations, there was a predominance of populations with 20 or fewer genets. A distribution such as this was also found by Weldy et al. (1996) in a survey of all known populations of *Cypripedium kentuckiense* C. F. Reed. The highly skewed distributions were hypothesized to be a consequence of slow population growth rates via sexual reproduction. This hypothesis seems a likely explanation for the typically small population sizes of southern *C. parviflorum* populations which have patchy distributions and may be more isolated than northern populations. Asexual reproduction via rhizomes is common in the southern populations visited in this study. Ellstrand and Roose (1987) have suggested that even plants that reproduce predominantly through asexual means may maintain high levels of genetic diversity. Based on data from 27 species which utilize some form of clonal propagation, they found

populations generally consisted of several distinct genotypes, and the genetic structure of these clonal populations could be as complex as more sexually reproducing populations. Clonal populations, they propose, are able to maintain at least intermediate levels of genetic diversity via small amounts of gene flow and/or mutations which generate variable genotypes (Ellstrand and Roose, 1987). Many of the southern populations which contain proportionately more asexual clumps than unique genotypes may maintain much of their genetic variation in this way. The high genetic identity values and low levels of among population differentiation in the species reflect the presence of at least limited amounts of current or historical gene flow. Similar to *C. parviflorum*, other species employing a combination of clonal and sexual reproduction exhibit high levels of diversity, significant levels of differentiation among populations, and multiclonal genotypes within populations (e.g., Eckert and Barrett, 1993; McClintock and Waterway, 1993).

The observed differences in levels of genetic variation between northern and southern areas may have been caused by either an increase in novel genetic variation in northern areas relative to southern areas, a loss in variation in the south relative to the north, or both. Although these data cannot definitively rule out any one of these hypotheses, the data suggest that there has been a loss of allelic diversity at the population level in the south. This conclusion is supported by the absence of widespread and unique alleles in the northern areas. Generally, northern populations and southern populations share the same suite of common alleles. Even many of the uncommon alleles (e.g., those found in six or fewer populations such as PGM-1b, PGM-1d, PGM-2a, IDH-1c, GDH-1b, and GOT-2a) can be found in northern as well as southern populations that are separated by large distances (e.g., PGM-1d was found in MI, VA, and GA). Very few alleles are

unique to either northern or southern states. Only four rare alleles were found exclusively in the north (i.e., MI; e.g., PGM-1a, SKD-1c, ADH-1a, and GDH-1a), and these were found in only one or two populations each. Likewise, four other alleles were found in the south, but not in the north [PGM-2d (VA), PGD-1a (MO), PGI-1c (VA), and GDH-1f (MO)]. These alleles were also rare and confined to one or two populations each. Therefore, the disparity in variation among the north and south is apparently not due to an increase in novel variation in the north, but rather to a decrease in overall genetic variation at the population level in the south.

The significantly smaller number of individuals found in southern *pubescens* populations compared to northern *pubescens* populations may account for the lower levels of genetic variation also seen in southern *pubescens* populations. Both alleles per locus and percent polymorphic loci are significantly correlated with population size (i.e., the number of genets) for all *pubescens* populations ($r = .68$ and $r = .31$, respectively; Table 17). This suggests that population size is directly related to the level of genetic variation maintained by *pubescens* populations. Similarly, the small flowered varieties (i.e., *parviflorum* and *makasin*) show a significant relationship between population size and alleles per locus ($r=0.70$; Table 17). Expected heterozygosity was not found to be significantly correlated with genet size in either group. These results are consistent with the theoretical findings of Nei et al. (1975) and Maruyama and Fuerst (1985). These researchers have investigated the effects of genetic bottlenecks on the number of alleles per locus, percent polymorphic loci, and heterozygosity. Their findings suggest that of the three statistics, heterozygosity should be affected least by severe genetic drift events.

Although the levels of genetic diversity are very different for northern and southern

C. parviflorum populations, they partition this variation in much the same way,

maintaining approximately 81% and 79% of the variation within populations, respectively.

These values may reflect the dispersal ability of this species rather than any historical

effects of colonization. The wind dispersed seeds and insect mediated pollination may

promote high levels of gene flow, low levels of population differentiation, and high levels

of genetic similarity. Hamrick et al. (1991) found a correlation between gene flow

potential and pollination and seed dispersal mechanisms. Species pollinated by animals

and having wind dispersed seeds exhibited lower levels of among population variation than

species with other combinations of traits. Additionally, plants with similar life history

traits to *C. parviflorum* exhibit similar Gst values. For example, other herbaceous

perennials maintain on average 77% of the variation within populations while outcrossing,

animal-pollinated species maintain 80% of the variation within populations (Hamrick and

Godt, 1989).

Taxonomic Implications

The morphological and isozyme data presented do not congruently resolve the

taxonomic ambiguity exhibited by the *C. parviflorum* species complex. While varieties

makasin and *pubescens* have clear isozyme and morphological differences, a taxonomic

separation between vars. *parviflorum* and *makasin* is supported only by the isozyme data.

Additionally, varieties *parviflorum* and *pubescens* are morphologically distinct, but are not

highly isozymically distinct.

Like *C. parviflorum*, other taxa exhibit significant morphological variability with

little divergence of isozymes (e.g., Lowrey and Crawford, 1985; Crawford and Steussy,

1987; Heywood and Levin, 1984; Freiley, 1993). Heywood and Levin (1984) were unable

to detect morphological variants or chromosomal races in *Gaillardia pulchella* on the

basis of allele frequencies, and caution that allozymes are not always indicative of

evolutionary divergence which may have occurred in the genetic composition of other

traits such as morphology, chemical composition, or chromosomal rearrangements.

Freiley (1993) also found discrepancies between morphological and genetic data sets in

the subspecific classification of *Haplopappus gracilis* (Nutt.) Gray. He proposed that

"insufficient time has elapsed since the derivation of the species for equivalent

differentiation at isozyme loci" even though "directional selection has acted to cause

substantial ecotypic differentiation among populations" (Freiley, 1993). The high

percentage of alleles that are shared between varieties, the paucity of ubiquitous alleles

unique to a variety, and the high genetic similarity among all three varieties suggests that

these taxa have recently separated or have experienced extensive secondary contact. There

has been little divergence in the allele frequencies surveyed, but sufficient levels of

morphological divergence between at least two varieties within the species have occurred.

This would account for the morphological distinctness between the small flowered

varieties (i.e., *parviflorum* and *makasin*) and *pubescens*.

Sympatric populations in which the varieties do not appear to be introgressing

might provide the clearest picture of the taxonomic relationships among the varieties.

Three such sympatric pairs of populations are included in this study. Two of the sets (Y/Z

and BB/AA) consist of *parviflorum/pubescens* individuals and one set (CC/DD) is made

up of *makasin/pubescens* individuals. In each of these populations, plants were readily

discernible as a small flowered variety or the large flowered variety. Although plants of

different varieties were not interspersed with one another in populations Y/Z and BB/AA, the populations were within approximately 20 meters of one another with no geographical or physical separation between them. Individuals of both morphologies were randomly intermixed in populations CC and DD. The habitats appeared equivalent with the exception that in populations AA and BB, the *pubescens* individuals were growing on land slightly more sloped than the *parviflorum* individuals. Both the morphological data and isozyme data indicate that populations Y/Z and BB/AA comprise distinct genetic entities which do not appear to introgress in these populations (Figs. 6,7). In each of these pairs of populations there is significant allele frequency heterogeneity over five and six loci, respectively (Table 13). This suggests that: 1) intervarietal gene flow is limited, 2) there is selection against the hybrids, or 3) one of the varieties has recently colonized the population with subsequent intervarietal reproduction not yet evident. While it is not possible to rule out the former two hypotheses, the latter does not seem likely. The sympatric nature of populations Y and Z was documented more than 10 years ago by Atwood (Tom Patrick, pers. comm.). He too was unable to find any hybrid individuals, and thereby regarded them as distinct species (Atwood, 1985). Likewise, Case first discovered populations CC and DD in 1987, and has visited these populations regularly. She too, has been unable to find any morphological intermediates in this population (Case, pers. comm.). It should be noted, however, that while many such sympatric populations occur in Michigan and elsewhere, there are also sympatric populations that show clear patterns suggestive of introgression (Wallace, pers obs.; Case, pers. comm.).

Although populations CC and DD have seven loci with significantly heterogeneous allele frequencies, there is an unusual finding which suggests gene flow may have occurred

in the past. One of the rarer alleles, GOT-2a, is found in both sympatric populations of *pubescens* and *makasin* (CC and DD). This allele was also found in other *pubescens* populations, but it was not detected in *parviflorum* populations or any other *makasin* populations. Although populations CC and DD are distinctly separate in the PCA plot of morphological characters (Fig. 5), population DD is contained within the central group of *parviflorum* and *pubescens* populations. This indicates a morphological similarity of population DD (var. *pubescens*) to populations of a small flowered variety. However, populations K and M, also northern *pubescens* populations and geographically close to population DD, are located in this central region as well. In the UPGMA based upon Average Taxonomic Distance (Fig. 6) populations CC and DD cluster with their respective varieties and away from one another. Case (1993) also recently studied populations CC and DD and found genetic patterns consistent with the possibility of restricted gene flow. She found four loci that exhibited significant allele frequency heterogeneity between populations. My results are consistent with her findings at these same four loci (TPI-2, GOT-2, PGD, and SKD). Consequently, it is highly likely that these and the other sympatric populations are behaving as distinct species which have restricted or no gene flow among them. The low frequency of GOT-2a in populations CC and DD may be the result of a few successful hybridization events between *makasin* and *pubescens* individuals. However, it appears that the majority of hybridization attempts are unsuccessful.

Although the varieties are probably genetically compatible in some of these sympatric sites (Newhouse, 1976; Atwood, 1985), they are rarely found in hybrid form. In the three sets of sympatric populations surveyed, no hybrid morphologies were

observed. Furthermore, only one population (population X) out of 30 contained

intermediate morphologies which were difficult to classify. Some populations, however,

suggest that introgression has occurred. In a review of the literature, Howard (1993) has

also found evidence of many species of animals and plants that appear to be reproductively

isolated in some areas of sympatric contact and appear to hybridize in other areas. Similar

observations have led Grant (1994) to hypothesize that character displacement is

commonly the result of competition between sympatric taxa and selection of ecological or

reproductive character(s) which results in floral isolation and divergence of taxa in areas

of sympatry. The taxa are forced to compete and in doing so develop different

characteristics which eliminate competition for the same resource. Howard (1993) views

reproductive character displacement as an observable pattern which may be the result of

either reinforcement or competition between taxa. Reinforcement, according to Howard

(1993), is the evolution of prezygotic reproductive isolating mechanisms in zones of

overlap or hybridization in response to selection against hybrid individuals. Howard

acknowledges that it may be difficult to determine which of these processes is operating in

sympatric populations, but the outcome of both may be reproductive character

displacement which is observable in sympatric populations. If these processes are

occurring in sympatric populations of C. parviflorum, we might expect to see evidence of

reproductive character displacement. For example, reproductive character displacement

has been proposed to explain why floral characters (e.g., color and size) in several plant

species differ greater when the species are in sympatry than when they are in allopatry

(e.g., Armbruster et al., 1994; Levin, 1985; Whalen, 1978). Levin (1985) found that

populations of Phlox drummondii Hook have pink corollas when they occur allopatrically

with the related pink flowered *Phlox cuspidata* Scheele and red corollas when the species

occur sympatrically. In a similar study, Whalen (1978) found differences in the size of

flowers of several *Solanum* species when they occur sympatrically. Both of these

characters may be related to pollinator differences, the authors hypothesize, which could

act to keep the species distinct even if they are genetically compatible. Based upon the

suggestion of Nilsson (1979) that pollinator size exerts a strong selective force upon

slipper size in European *Cypripedium calceolus* species, Atwood (1985) has proposed

that pollinator size may also be a selective force influencing slipper size in *pubescens* and

parviflorum. Although individuals of each of the sympatric populations were readily

assignable to one or the other variety, the floral character differences among varieties did

not seem to differ by larger degree than they did in allopatric comparisons. Further study

of the morphology and ecology of sympatric and allopatric populations is needed to assess

the presence of reproductive character displacement and its potential evolutionary

mechanism.

In summary, the formal recognition of *C. parviflorum* should be limited to two

taxa, distinct at the varietal level. Morphologically, plants from throughout the eastern

United States can be divided into two statistical groups- one with large slippers and

yellowish-green overcoloring (i.e., var. *pubescens*) and one with small slippers and

reddish-purple overcoloring (var. *parviflorum*). However, there is no quantitative or

qualitative indication that *parviflorum* differs significantly from *makasin* morphologically.

Although the isozyme data indicate differences in allele frequencies among *parviflorum*

and *makasin* populations, these differences also occur in northern vs. southern populations

of var. *pubescens*. Therefore, it is possible that relatively recent patterns of gene flow are

more responsible than historical phylogenetic patterns for the isozyme dissimilarity of *parviflorum* and *makasin*. This is further supported by the widespread occurrence of common and uncommon alleles shared by both taxa, and the lack of any ubiquitous varietal specific alleles. Lastly, the recognition of var. *makasin* based on allelic data would also necessitate the recognition of a strictly northern variety of *pubescens*. Neither of these recognitions would be supported by the morphological characters in this study.

Conservation Implications

Cypripedium parviflorum is facing several threats. Factors such as competition from other species, herbivory, natural successional and human-induced destruction of suitable habitat are well documented forces that contribute to the extinction of a population (e.g., Frankel and Soule, 1981; Soule, 1983; Lande and Barrowclough, 1987). A catastrophic event can wipe out an entire population very quickly, drawing attention to the necessity of preserving Lady Slipper habitat in its natural state. Gradual shrinking of suitable habitat or destruction of habitat bordering a population may potentially limit gene flow by interfering with pollinator activity or seed dispersal. Although seeds of *C. parviflorum* are wind dispersed and are expected to travel long distances, they may not land in suitable habitat to initiate colonization or migrate to an existing population. Another potential threat may be the genetic effects of small population size. Throughout its range, southern populations are probably at the greatest risk of suffering from genetic drift and inbreeding due to fewer, smaller populations and greater isolation of these populations. One population (C) exhibited significant deviations from Hardy-Weinberg equilibria consistent with inbreeding. Other populations not sampled and smaller in size may also be affected. Small population size has been recognized to increase the potential

for genetic drift and inbreeding which, in some species, can lead to a decrease in fitness via inbreeding depression. This could further lead to increased vulnerability to pathogens, and the inability of plants to respond to environmental variability (Ledig, 1986).

This study indicates that southern populations have significantly lower levels of genetic variation than northern populations, but still maintain moderately high levels of variation within populations. Furthermore, this research suggests that the lowered level of variation in southern populations is a result of small population sizes and corresponding loss of alleles. Management practices should focus on the maintenance of present levels of morphological and genetic variation by promoting population expansion and preserving available habitat.

Management practices such as prescribed burning and fencing may be beneficial in many populations threatened by competing species and deer. Recently, some organizations have begun to experiment with prescribed burns. This process, once occurring naturally on prairie lands, is necessary for germination and growth in some species, and for *C. parviflorum* may eliminate many of the weedy annuals, short-lived perennials, and canopy species that compete for space, nutrients, and sunlight. Population B experienced a controlled burn in the Spring of 1994 (before *C. parviflorum* came up; Schuette, pers. comm.). When I visited this population in 1995, there were approximately 75 plants scattered throughout an area of 150 feet. The majority of the population consisted of flowering adult individuals and a smaller number of nonflowering juveniles and very young plants that may have been new recruits. Additionally, clonal reproduction was not as common in population B as it was in other southern populations. While I

cannot be sure that the burn alone improved conditions for this population, it does seem possible that it at least aided in opening up the area for new growth of *C. parviflorum*.

Browsing by deer is also a serious threat to many populations on protected land. Lady's Slippers seem to be a special treat for deer, and an entire population can be stripped of their flowers very quickly (Wallace, pers. obs.; Case, pers. comm.). Consequently, sexual reproduction for that year is arrested. Through herbivory, the size of a reproductive population may be decreased, thereby limiting gene flow and contributing to genetic drift. Protecting these populations by enclosing them in fencing during the period of flowering and seed set could be an easy and effective management practice to prevent unwanted browsing by deer. Some organizations have begun to experiment with this method as well (Schuette, pers. comm.). In theory, this could be a great tool for stabilizing and possibly increasing the size of populations which have recently been hit hard by an exploding deer population. Further research in this area may be necessary to fully evaluate all of the factors involved in the growth and maintenance of populations.

Summary and Conclusions

Sheviak's (1994) proposal of three varieties of Yellow Lady's Slipper is not supported in this study. Univariate, principal components, and UPGMA cluster analyses of morphological characters show that vars. *parviflorum* and *makasin* are largely indistinguishable from one another. However, these varieties are morphologically distinct from southern as well as northern populations of var. *pubescens*. The isozyme data, in contrast, reveal no differences among *parviflorum* and *pubescens* populations, but show a common clustering region of *makasin* populations. This was apparently due to relatively

high levels of genetic variation in *makasin* populations, which resulted from allele

frequency differences rather than the presence of unique alleles. Although the *makasin*

populations clustered together, a very high level of among population variation in var.

makasin prevents definitive identification of this taxon based on allele frequency data. All

varieties in the complex appear to be recently diverged. This is supported by a lack of

variety specific alleles and the large geographic distances that separate populations which

often share the same rare alleles. Lastly, only 3% of the total species level variation

resides among the varieties.

The results from this study are largely consistent with previous conclusions based

on analyses of northern populations of vars. *pubescens* and *makasin* by Case (1993).

However, the close isozyme similarity of vars. *parviflorum* and *pubescens* in the south

was unexpected based on previous results for northern populations. Furthermore,

populations of var. *pubescens* and var. *parviflorum* in the south were significantly less

variable than populations in the northern areas. It is suggested that northern areas were

particularly suitable for the colonization and maintenance of large populations as glaciers

retreated. Southern areas, however, may have been vegetated more heavily, containing

habitats less conducive to large population sizes and interpopulation gene flow. This

situation would have created a loss of alleles due to genetic drift in the south relative to

the northern areas.

The study of sympatric populations and conservation strategies represent areas of

needed future research on this species. Specifically, studies that focus on the mechanisms

of isolation in sympatric populations may reveal important evolutionary mechanisms that

may have been responsible for the evolution of the varieties within this complex. In

addition, other types of molecular data, such as cpDNA restriction site data, may provide additional insight into the phylogenetic relationships among these varieties. Lastly, additional population biology studies are needed before effective population management plans can be applied. Specific areas of applicable research include the effect of pollinator behavior on gene flow, ecological work that focuses on habitat requirements, and demographic analyses that may indicate what life history stages critically affect population growth rates. These studies are especially relevant to southern populations.

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TABLE 1. Reference letters, sample size (N), location, and general geographic region (S= southern, N= northern) of populations of *C. parviflorum* vars. *pubescens* (**PUB**), *parviflorum* (**PARV**), and *makasin* (**MAK**) based upon initial morphological determination. Pure site populations included individuals that were easily classified into one of the varieties, and no intermediate morphologies were observed. Mixed sites included populations of intermediate morphologies as well as sympatric populations. Only one population (X) included both intermediate morphologies and distinct forms and is labeled as "Both". The remaining mixed site populations (Y, Z, AA, BB, CC, DD) were in sympatry but no intermediate morphologies were found. These sites are indicated as "SYM" followed by the name of the other sympatric population. Sites for which morphological measurements are also available are indicated by an asterisk (*).

POPULATION	TAXON	N	LOCATION	GEOGRAPHIC REGION
Pure Sites				
*A	PUB	13	Wayne Co., MO	S
B	PUB	20	Lincoln Co., MO	S
*C	PUB	9	Lincoln Co., MO	S
*D	PUB	3	Nelson Co., VA	S
E	PUB	8	Nelson Co., VA	S
*F	PUB	3	Nelson Co., VA	S
G	PUB	23	Sevier Co., TN	S
*H	PUB	29	Noble Co., IN	N
*I	PUB	2	Noble Co., IN	N
*J	PUB	19	James City Co., VA	S
*K	PUB	27	Mackinac Co., MI	N
*L	PUB	20	Emmet Co., MI	N
*M	PUB	20	Presque Isle Co., MI	N
*N	PUB	20	Bullitt Co., KY	S
*O	PARV	13	Shannon Co., MO	S
*P	PARV	16	Texas Co., MO	S
*Q	PARV	19	Haswell Co., MO	S
*R	PARV	17	Oregon Co., MO	S
*S	PARV	20	Habersham Co., GA	S
*T	PARV	10	Steuben Co., IN	N
*U	PARV	40	Cherokee Co., OK	S
*V	MAK	26	Chippewa Co., MI	N
*W	MAK	20	Presque Isle Co., MI	N

Table 1, continued

Mixed Sites

*X	PARV/PUB (Both)	22	Carter Co., MO	S
*Y	PARV (Sym/Z)	10	Union Co., GA	S
*Z	PUB (Sym/Y)	19	Union Co., GA	S
*AA	PUB (Sym/BB)	10	Nelson Co., VA	S
*BB	PARV (Sym/AA)	2	Nelson Co., VA	S
*CC	MAK (Sym/DD)	33	Chippewa Co., MI	N
*DD	PUB (Sym/CC)	22	Chippewa Co., MI	N

Table 2. Reference letters, morphological taxon, and sample size (N) for populations from Case's (1993) study. The population names that Case (1993) used are cross-referenced with those names used in this study. PUB= *pubescens*; MAK= *makasin* (= *parviflorum* in Case); BOTH= both morphological taxa (i.e., *makasin* and *pubescens*) and intermediate morphologies present; HYB= only individuals with intermediate morphologies present. All populations are located in a northern geographic region as defined in the text.

POPULATION	TAXON	CROSS-REFERENCE (Case, 1993)	N
EE	PUB	E	20
FF	PUB	F	20
GG	PUB	G	20
HH	PUB	B	20
II	PUB	C	20
JJ	PUB	D	20
KK	PUB	A	20
LL	MAK	H	88
MM	MAK	I	22
NN	MAK	J	47
OO	MAK	K	50
PP	BOTH	R	20
QQ	BOTH	T	10
RR	BOTH	U	20
SS	HYB	S	19
TT	HYB	W	20

Table 3. Results of Dunn's nonparametric multiple comparisons test (Zar, 1996) among varieties *pubescens* (PUB), *parviflorum* (PARV), and *makasin* (MAK) for 15 morphological characters. The mean ± standard error, range (in parentheses), and sample size (N) for each measurement of each variety are listed. All measurements are in cm. Means followed by the same letter are not significantly different, and means followed by different letters are significantly different at $p \leq 0.005$ (*) or $p \leq 0.001$ (**). Petal width was not tested for significance (see text).

Morphological Character	PUB	PARV	MAK
Petal Length	7.07±0.228a** (4.4-10.5) N=42	5.27±0.147b** (3.0-7.25) N=43	4.14±0.182c** (3.25-4.5) N=7
Dorsal Sepal Width	2.20±0.054a** (1.5-2.9) N=42	1.77±0.048b** (1.25-2.5) N=43	1.40±0.049c** (1.2-1.6) N=7
Lateral Sepal Length	4.77±0.155a** (3.1-6.75) N=41	3.98±0.130b** (2.0-5.75) N=42	2.93±0.190c** (2.4-3.7) N=7
Orifice Length	0.90±0.034a (0.4-1.5) N=42	0.68±0.039b** (0.3-1.3) N=43	0.89±0.144a (0.6-1.7) N=7
Staminode Length	1.23±0.031a** (0.75-1.7) N=42	1.03±0.029b (0.75-1.25) N=43	1.04±0.065b (0.8-1.3) N=7
Lateral Sepal Width	1.93±0.058a** (1.2-2.5) N=41	1.35±0.047b (0.75-2.0) N=42	1.20±0.072b (0.9-1.50) N=7
Slipper Length	3.77±0.090a** (2.5-5.8) N=42	2.49±0.079b (1.25-3.5) N=43	2.51±0.080b (2.2-2.8) N=7

Table 3, continued

Slipper Width	2.16±0.053a** (1.2-3.0) N=42	1.36±0.057b (0.5-2.25) N=43	1.49±0.059b (1.2-1.7) N=7
Orifice Width	1.02±0.033a** (0.5-1.5) N=42	0.79±0.037b (0.25-1.5) N=43	0.73±0.036b (0.6-0.8) N=7
Height	45.62±1.311a (27.8-60.5) N=42	44.53±1.456a (26.0-61.0) N=43	31.81±1.196b** (29.0-35.5) N=7
Leaf Length	14.77±0.403a (9.0-21.0) N=42	14.42±0.501a (4.25-20.0) N=43	10.40±0.373b** (9.5-11.9) N=7
Leaf Width	7.12±0.324a (2.8-10.5) N=42	7.27±0.310a (2.75-11.5) N=43	3.26±0.373b** (1.9-4.7) N=7
Dorsal Sepal Length	5.53±0.187a (3.5-7.5) N=42	4.47±0.125a (2.5-6.0) N=43	3.21±0.150b** (2.7-3.7) N=7
Number of Twists per Lateral Petal	3.61±0.241a (1.0-7.0) N=42	3.85±0.321a (0.5-7.0) N=43	3.29±0.286a (2.0-4.0) N=7
Petal Width (No Test)	0.66±0.024 (0.5-1.0) N=42	0.49±0.020 (0.25-1.0) N=43	0.44±0.020 (0.4-0.5) N=7

Table 4. Character loadings for each of the floral morphological traits and percent of variation explained by the first three principal component axes. See also Figure 4.

FLORAL CHARACTER	PRINCIPAL COMPONENT AXIS		
	1	2	3
Twists/Petal	0.321	-0.749	-0.417
Staminode Length	0.748	-0.025	0.056
Petal Length	0.913	-0.286	0.074
Petal Width	0.658	0.441	0.264
Dorsal Sepal Length	0.887	-0.301	0.172
Dorsal Sepal Width	0.867	0.067	0.091
Lateral Sepal Length	0.828	-0.347	0.169
Lateral Sepal Width	0.845	0.141	0.224
Slipper Length	0.907	0.046	-0.001
Slipper Width	0.806	0.325	-0.154
Orifice Length	0.513	0.310	-0.708
Orifice Width	0.711	0.205	-0.310
Percent of Variation	**59.23**	**10.99**	**8.25**

Table 5. Character loadings of first three principal component axes plotted in Figure 5. The characters represent mean population measurements. The percent variation explained by each axis is listed under each axis column.

MORPHOLOGICAL CHARACTER	PRINCIPAL COMPONENT AXIS		
	1	2	3
Height	0.687	0.605	-0.175
Twists/Petal	0.314	0.479	0.727
Staminode Length	0.848	-0.176	0.289
Leaf Length	0.667	0.448	-0.299
Leaf Width	0.667	0.539	-0.302
Petal Length	0.932	0.002	0.194
Petal Width	0.694	-0.384	-0.400
Dorsal Sepal Length	0.928	0.129	0.026
Dorsal Sepal Width	0.956	-0.098	-0.115
Lateral Sepal Length	0.935	0.172	0.060
Lateral Sepal Width	0.930	-0.148	-0.023
Slipper Length	0.887	-0.237	0.273
Slipper Width	0.832	-0.422	0.167
Orifice Length	0.659	-0.284	-0.151
Orifice Width	0.768	-0.145	-0.112
Percent of Variation	**63.66**	**11.20**	**7.85**

Table 6. Genetic Identity and Taxonomic Distance values for interspecific and intraspecific comparisons of vars. *parviflorum* (PARV), *pubescens* (PUB), and *makasin* (MAK). A) Nei's Genetic Identities for intravarietal and intervarietal comparisons of populations surveyed in this study; B) Nei's Genetic Identities for populations surveyed in this study combined with populations studied by Case (1993); and C) Average Taxonomic Distance based on morphological data between all pairwise comparisons of populations from the present study.

A)

| | INTRAVARIETAL | | | INTERVARIETAL | |
	Mean	Range		Mean	Range
PUB	0.965	0.825-1.0	PUB/PARV	0.963	0.881-1.0
PARV	0.965	0.903-1.0	PUB/MAK	0.901	0.770-0.949
MAK	0.943	0.909-0.977	PARV/MAK	0.902	0.816-0.957
Unweighted Mean	**0.958**	**0.825-1.0**	**Unweighted Mean**	**0.922**	**0.770-1.0**

B)

| | INTRAVARIETAL | | | INTERVARIETAL | |
	Mean	Range		Mean	Range
PUB	0.969	0.820-1.0	PUB/PARV	0.974	0.869-1.0
PARV	0.979	0.933-1.0	PUB/MAK	0.898	0.635-1.0
MAK	0.914	0.787-0.991	PARV/MAK	0.892	0.748-0.975
Unweighted Mean	**0.954**	**0.787-1.0**	**Unweighted Mean**	**0.921**	**0.635-1.0**

C)

| | INTRAVARIETAL | | | INTERVARIETAL | |
	Mean	Range		Mean	Range
PUB	1.029	0.434-1.965	PUB/PARV	1.566	0.800-2.876
PARV	1.026	0.373-1.683	PUB/MAK	1.703	0.913-2.525
MAK	0.616	0.557-0.688	PARV/MAK	1.103	0.693-1.692
Unweighted Mean	**0.890**	**0.373-1.965**	**Unweighted Mean**	**1.457**	**0.693-2.876**

Table 7. Allele frequencies weighted according to the number of individuals per variety for all loci. N= number of individuals sampled over all loci.

VARIETY	PUB N=267	PARV N=147	MAK N=79
LOCUS			
PGM-1			
a	0.004	___	___
b	___	0.014	0.006
c	0.888	0.969	0.994
d	0.020	0.003	___
e	0.088	0.014	___
PGM-2			
a	___	0.010	___
b	0.254	0.269	0.164
c	0.740	0.721	0.836
d	0.006	___	___
IDH-2			
a	0.966	0.983	0.842
b	0.032	0.017	0.152
c	0.002	___	0.006
SKD-1			
a	0.290	0.218	0.570
b	0.710	0.782	0.380
c	___	___	0.050
PGD-1			
a	0.002	___	___
b	0.893	0.986	0.715
c	0.024	___	0.051
d	0.081	0.014	0.234
PGI-1			
a	0.104	0.252	0.031
b	0.868	0.728	0.899
c	0.002	___	___
d	0.026	0.020	0.070

Table 7, continued

SOD-1			
a	1.000	1.000	1.000
ADH-1			
a	0.004	___	___
b	0.153	0.067	0.006
c	0.843	0.933	0.994
GDH-1			
a	___	___	0.114
b	0.004	0.061	0.253
c	0.962	0.872	0.431
d	0.021	0.027	0.145
e	0.013	0.037	0.057
f	___	0.003	___
GOT-1			
a	1.000	1.000	1.000
GOT-2			
a	0.017	___	0.006
b	0.354	0.255	0.228
c	0.011	0.072	0.025
d	0.618	0.673	0.741
TPI-1			
a	0.073	0.020	0.183
b	0.927	0.980	0.817
TPI-2			
a	0.908	0.990	0.405
b	0.092	0.010	0.595

Table 8. Measures of diversity for *C. parviflorum* vars. *parviflorum*, *pubescens*, and *makasin*. Percent polymorphic loci (P), alleles per locus (A), observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}) are given for each population. The average percent polymorphic loci and alleles per locus are given at the population (P_P, A_P), varietal (P_v, A_v), and species (P_s, A_s) levels. PUB= *pubescens*; PARV= *parviflorum*; MAK= *makasin*; SPECIES= all populations included. Population letters are referenced in Table 1.

Population	P	A	H_{OBS}	H_{EXP}
A	0.462	1.5	0.142	0.122
B	0.615	1.8	0.161	0.203
C	0.538	1.5	0.068	0.201
D	0.231	1.2	0.103	0.123
E	0.462	1.5	0.173	0.129
F	0.385	1.5	0.205	0.190
G	0.615	1.7	0.127	0.138
H	0.692	1.8	0.144	0.152
I	0.385	1.4	0.308	0.231
J	0.769	1.9	0.198	0.227
K	0.846	2.2	0.265	0.256
L	0.538	1.5	0.192	0.178
M	0.769	2.1	0.246	0.222
N	0.462	1.5	0.176	0.145
O	0.385	1.5	0.095	0.116
P	0.462	1.5	0.106	0.124
Q	0.462	1.5	0.150	0.123
R	0.308	1.4	0.181	0.135
S	0.538	1.7	0.150	0.156
T	0.538	1.6	0.269	0.213
U	0.231	1.3	0.092	0.098
V	0.846	2.2	0.222	0.258
W	0.692	2.0	0.204	0.251
X	0.462	1.5	0.210	0.160
Y	0.538	1.5	0.214	0.246
Z	0.615	1.6	0.113	0.115
AA	0.154	1.2	0.077	0.060
BB	0.385	1.4	0.231	0.205
CC	0.692	2.1	0.221	0.250
DD	0.769	2.0	0.169	0.214

Table 8, continued

	P_P (P_V)	A_P (A_V)		
PUB	0.555 (0.846)	1.65 (2.77)	0.169	0.171
PARV	0.418 (0.846)	1.50 (2.38)	0.165	0.157
MAK	0.769 (0.846)	2.10 (2.46)	0.216	0.253
	P_P (P_S)	A_P (A_S)		
SPECIES	0.528 (0.846)	1.64 (3.23)	0.174	0.175

Table 9. Number and average frequency of alleles unique to a single variety and the number and average frequency of alleles unique to populations within each variety (private alleles). The number and percentage of alleles shared between varieties is also reported. PUB = *pubescens*; PARV = *parviflorum*; MAK = *makasin*.

Variety/ Comparison	Number of Unique Alleles	Mean Frequency of Unique Alleles	Number of Private Alleles	Mean Frequency of Private Alleles	Total Number of Alleles Present	Number of Alleles Shared	Percentage of Alleles Shared
PUB	5	0.006	4	0.004	--	--	--
PARV	2	0.008	1	0.004	--	--	--
MAK	2	0.093	0	---	--	--	--
PUB/PARV	-	---	-	---	40	28	70
PUB/MAK	-	---	-	---	40	29	73
PARV/MAK	-	---	-	---	35	28	80

Table 10. Loci with significant deviations from Hardy-Weinberg equilibrium. For each locus, the significance level (P) and fixation index (F) are given. Deviations were considered statistically significant if $p \leq 0.05$. A positive fixation index indicates a deficiency of heterozygous individuals, and a negative index indicates an excess of heterozygous individuals. Populations are referenced in Table 1.

Population and Locus		P	F
A	GOT-2	0.034	-0.580
R	PGI-1	0.049	-0.545
S	GDH -1	<0.001	1.0
T	SKD-1	0.045	-0.818
U	GOT-2	0.001	0.547
Y	GDH-1	0.001	1.0
CC	GDH-1	0.004	0.581
DD	GOT-2	0.003	0.392
B	PGM-2	<0.001	0.900
	PGD-1	0.008	0.560
J	PGI-1	0.012	0.550
	TPI-1	0.034	0.604
V	IDH-2	0.049	0.424
	GDH-1	0.038	0.569
C	PGM-1	0.005	1.0
	PGM-2	0.003	1.0
	PGI-1	0.015	1.0

Table 11. Nei's diversity statistics for *C. parviflorum* vars. *parviflorum, pubescens,* and *makasin* averaged across all loci. H_T= the total amount of diversity; H_S= average amount of expected heterozygosity; D_{ST}= the absolute amount of variation distributed among populations; G_{ST}= the percent of variation distributed among populations relative to the total variation. Estimates are reported for each variety and for the species including only populations in this study (SPECIES). Diversity statistics are also reported for northern and southern populations of *makasin, parviflorum,* and *pubescens,* and for the species with the inclusion of Case's (1993) populations (COMBINED). See also Appendix 4 for single locus diversity statistics of each taxon and standard errors of loci. PUB= *pubescens;* PARV= *parviflorum;* MAK= *makasin.*

Taxon	H_T	H_S	D_{ST}	G_{ST}
PUB	0.199	0.160	0.039	0.196
PARV	0.182	0.147	0.035	0.192
MAK	0.280	0.248	0.032	0.114
SPECIES	0.213	0.165	0.048	0.224
COMBINED N. PUB	0.203	0.169	0.033	0.163
COMBINED S. PUB	0.173	0.123	0.039	0.225
COMINED PARV	0.148	0.123	0.024	0.162
COMBINED MAK	0.298	0.220	0.079	0.265
COMBINED SPECIES	0.215	0.167	0.049	0.228

Table 12. Individual allelic character loadings and percent of variation explained by the first three principal component axes. These principal components correspond to the plot in Figure 8.

LOCUS & ALLELE	PRINCIPAL COMPONENT AXIS		
	1	2	3
PGM-1a	0.169	0.081	-0.112
PGM-1b	-0.156	-0.080	0.197
PGM-1c	0.228	0.189	0.507
PGM-1d	-0.049	-0.128	-0.239
PGM-1e	-0.198	-0.129	-0.436
PGM-2a	-0.085	-0.240	0.106
PGM-2b	-0.220	0.133	-0.569
PGM-2c	0.282	-0.004	0.595
PGM-2d	-0.066	-0.182	-0.057
IDH-1a	-0.782	-0.006	-0.008
IDH-1b	0.770	0.096	0.009
IDH-1c	0.083	-0.384	-0.004
SKD-1a	0.573	-0.256	0.086
SKD-1b	-0.612	0.214	-0.098
SKD-1c	0.537	0.392	0.139
PGD-1a	-0.061	0.230	-0.184
PGD-1b	-0.490	-0.630	0.026
PGD-1c	0.336	-0.171	-0.216
PGD-1d	0.404	0.757	0.069
PGI-1a	-0.439	-0.385	0.506
PGI-1b	0.318	0.553	-0.442
PGI-1c	-0.064	-0.191	-0.048
PGD-1d	0.476	-0.362	-0.249
SOD0-1a	0.000	0.000	-0.000
ADH-1a	0.176	-0.137	-0.225
ADH-1b	-0.186	0.527	0.052
ADH-1c	0.180	-0.521	-0.044
GDH-1a	0.483	0.106	0.179
GDH-1b	0.480	0.089	0.054
GDH-1c	-0.762	0.155	-0.195
GDH-1d	0.604	-0.290	-0.198
GDH-1e	0.197	-0.342	0.636
GDH-1f	-0.102	-0.321	0.695

Table 12, continued

GOT-1a	0.000	0.000	-0.000
GOT-2a	0.151	0.089	-0.129
GOT-2b	0.043	-0.563	-0.687
GOT-2c	0.037	-0.437	0.574
GOT-2d	-0.067	0.670	0.553
TPI-1a	0.742	-0.191	-0.115
TPI-1b	-0.742	0.191	0.115
TPI-2a	-0.891	0.004	0.015
TPI-2b	0.891	-0.003	-0.015
Percent of Variation	**26.71**	**21.25**	**12.80**

Table 13. Results of Contingency X^2 test for heterogeneity among each of three pairs of sympatric populations (Y/Z, AA/BB, CC/DD). The significance level is reported for each polymorphic locus. Heterogeneity among populations is considered significant at $p \leq 0.05$ (*) or $p \leq 0.001$ (**). Loci that were not polymorphic are designated as NP.

Polymorphic Locus	Significance Level		
	Y/Z	AA/BB	CC/DD
PGM-1	0.040*	0.022*	0.013*
PGM-2	0.008*	0.022*	0.254
IDH-2	NP	NP	0.080
SKD-1	0.312	0.022*	<0.001**
PGD-1	0.133	0.206	<0.001**
PGI-1	0.001**	0.022*	0.008*
ADH-1	0.056	0.002*	NP
GDH-1	<0.001**	NP	<0.001**
GOT-2	0.001**	0.001**	<0.001**
TPI-1	0.090	NP	0.904
TPI-2	0.464	NP	<0.001**
Total Over All Loci	<0.001**	< 0.001**	<0.001**

Table 14. Correlation (r) between matrices of geographic distance, Average Taxonomic Distance based on morphology, and Nei's (1978) Unbiased Genetic Distance between all pairs of populations. The matrix correlation is equivalent to the normalized Mantel statistic (Z). The significance of the association between matrices was tested through 1000 random permutations and the probability reported is that of a random Z value being greater than or equal to the observed Z value. An association is significant at $p \leq 0.05$ (*).

Matrix Comparison	Correlation (r)	Significance Level(p)
Geographic Distance X Nei's Unbiased Genetic Distance	0.162	0.006*
Geographic Distance X Average Taxonomic Distance	0.003	0.431
Average Taxonomic Distance X Nei's Unbiased Genetic Distance	0.136	0.092

Table 15. Populations of southern and northern *pubescens* and corresponding population size (i.e., # of genets), sample size (N), alleles/locus (A), percent polymorphic loci (P), and expected heterozygosity (Hs) values. Means for each group are reported also. The results of a t-test for significance between the two groups are listed at the bottom for each variable.

POP	POP SIZE	N	A	P	Hs
SOUTH					
A	13	13	1.5	45.5	0.120
B	50	20	1.8	63.6	0.230
C	9	9	1.5	45.5	0.162
D	3	3	1.3	27.3	0.145
E	8	8	1.5	54.5	0.153
F	3	3	1.5	36.4	0.194
G	45	23	1.7	63.6	0.132
J	19	19	1.9	81.8	0.247
N	30	20	1.5	45.5	0.137
Z	22	19	1.7	72.7	0.136
AA	10	10	1.2	18.2	0.071
$\overline{X}$	19.27	13.36	1.6	50.4	0.157
NORTH					
H	70	29	1.7	63.6	0.143
I	2	2	1.4	36.4	0.212
K	550	27	2.0	81.8	0.260
L	180	20	1.5	54.5	0.206
M	900	20	2.0	72.7	0.236
DD	270	22	1.9	72.7	0.212
EE	40	20	1.5	54.5	0.159
FF	30	20	1.8	72.7	0.214
GG	75	20	1.6	63.6	0.217
HH	1000	20	1.7	63.6	0.168
II	2000	20	1.8	81.8	0.167
JJ	200	20	1.6	63.6	0.182
KK	200	20	1.6	54.5	0.140
$\overline{X}$	424.38	20	1.7	64.3	0.194
Significance Level	$p < 0.005$	not tested	$p < 0.001$	$p < 0.001$	$p < 0.001$

Table 16. Populations of *parviflorum* and *makasin* and corresponding population size (i.e., # of genets), sample size (N), alleles per locus (A), percent polymorphic loci (P), and expected heterozygosity (Hs) values. Means for each group are also reported. The results of t-tests for significant differences between the groups for each variable are reported at the bottom for all variables except percent polymorphic loci which was tested via a Kruskal-Wallis test.

POP	POP SIZE	N	A	P	Hs
PARVIFLORUM					
O	13	13	1.4	27.3	0.106
P	16	16	1.5	45.5	0.100
Q	19	19	1.5	36.4	0.098
R	17	17	1.2	18.2	0.075
S	20	20	1.7	54.5	0.154
T	10	10	1.6	54.5	0.209
U	50	40	1.2	18.2	0.092
Y	10	10	1.5	45.5	0.207
BB	2	2	1.4	36.4	0.197
$\overline{X}$	17.44	16.33	1.4	37.4	0.138
MAKASIN					
V	180	26	2.0	81.8	0.229
W	90	20	1.8	63.6	0.197
CC	270	33	1.8	63.6	0.229
LL	360	88	1.7	72.7	0.215
MM	50	22	1.8	72.7	0.250
NN	300	47	1.9	72.7	0.304
OO	300	50	2.0	81.8	0.222
$\overline{X}$	221.43	40.86	1.9	72.7	0.235
Significance Level	$p < 0.05$	not tested	$p < 0.001$	$p < 0.001$	$p < 0.001$

Table 17. Spearman's rank correlation between population size (i.e., # of genets) and alleles per locus (A), percent polymorphic loci (P), and expected heterozygostiy (Hs) for 24 *pubescens* populations and 17 *parviflorum* and *makasin* populations. The significance level of the correlation is also given immediately below the correlation coefficient for each variable. NS= correlation was not significant at $p \leq 0.05$.

	PUBESCENS	PARVIFLORUM/MAKASIN
A	0.68	0.70
	$p < 0.05$	$p < 0.05$
P	0.67	0.40
	$p < 0.05$	NS
Hs	0.31	0.17
	NS	NS
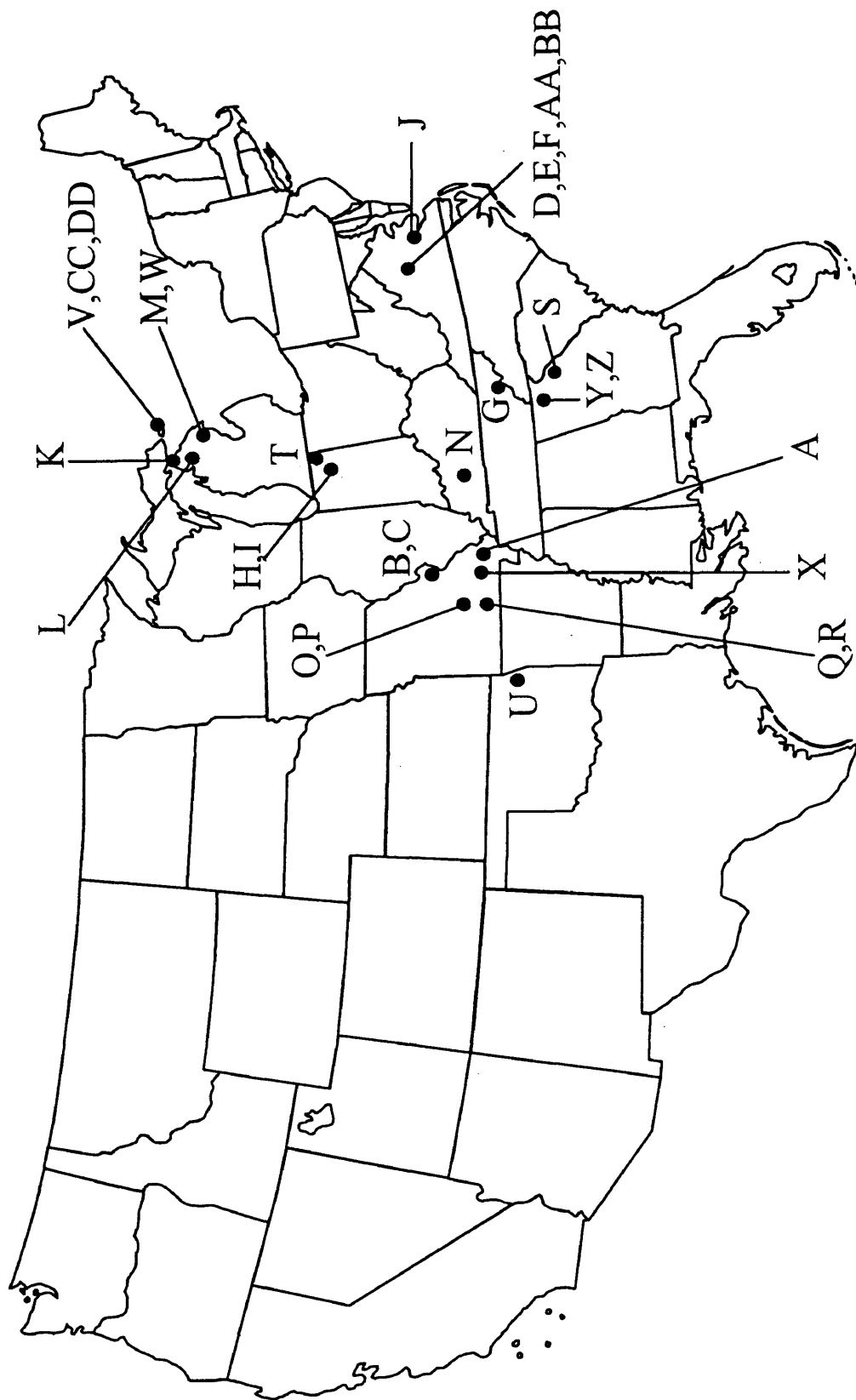
Figure 1. Map of *Cypripedium parviflorum* populations sampled for this study.
Letters are referenced in Table 1.
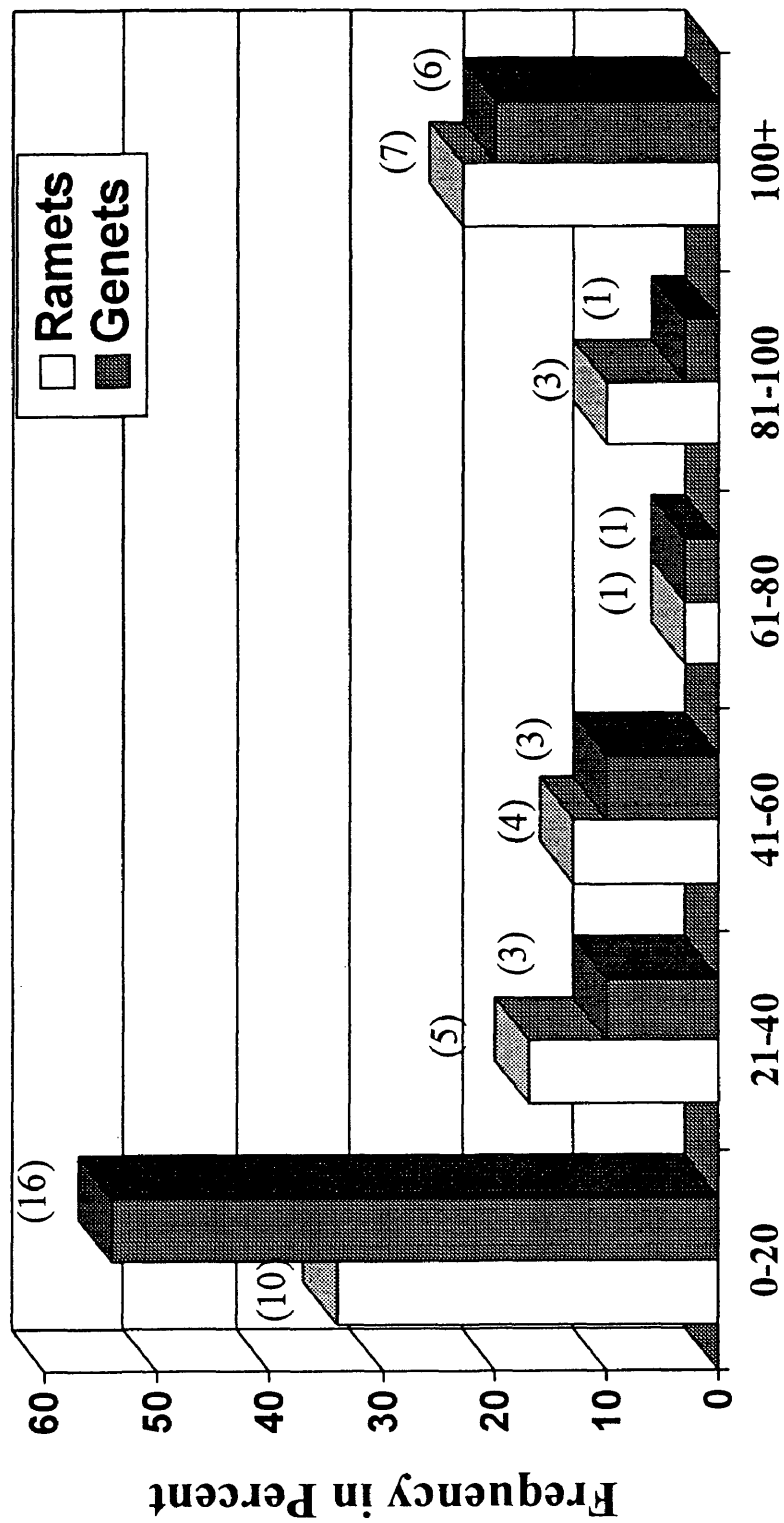
Figure 2. Frequency of ramets or genets found in *Cypripedium parviflorum* populations. The absolute number of populations for each category is in parentheses.
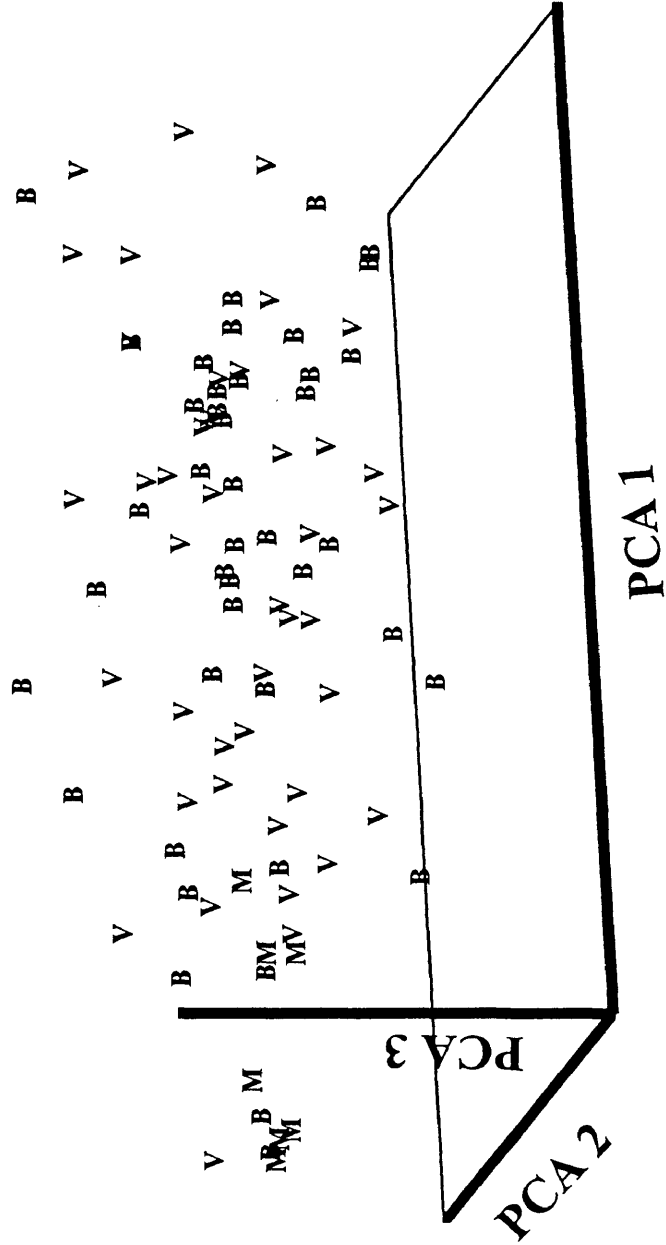
Figure 3. Three dimensional plot of the first three principal component axes resulting from vegetative morphological measurements of individuals. The first, second, and third axes account for 78.45%, 15.25%, and 6.29% of the variation, respectively. B= *pubescens*, V= *parviflorum*, M= *makasin*.

Figure 4. Three dimensional plot of the first three principal component axes resulting from floral morphological characters of individuals. The first, second, and third axes account for 59.23%, 10.99%, and 8.25% of the variation, respectively. B= *pubescens*, V= *parviflorum*, M= *makasin*.
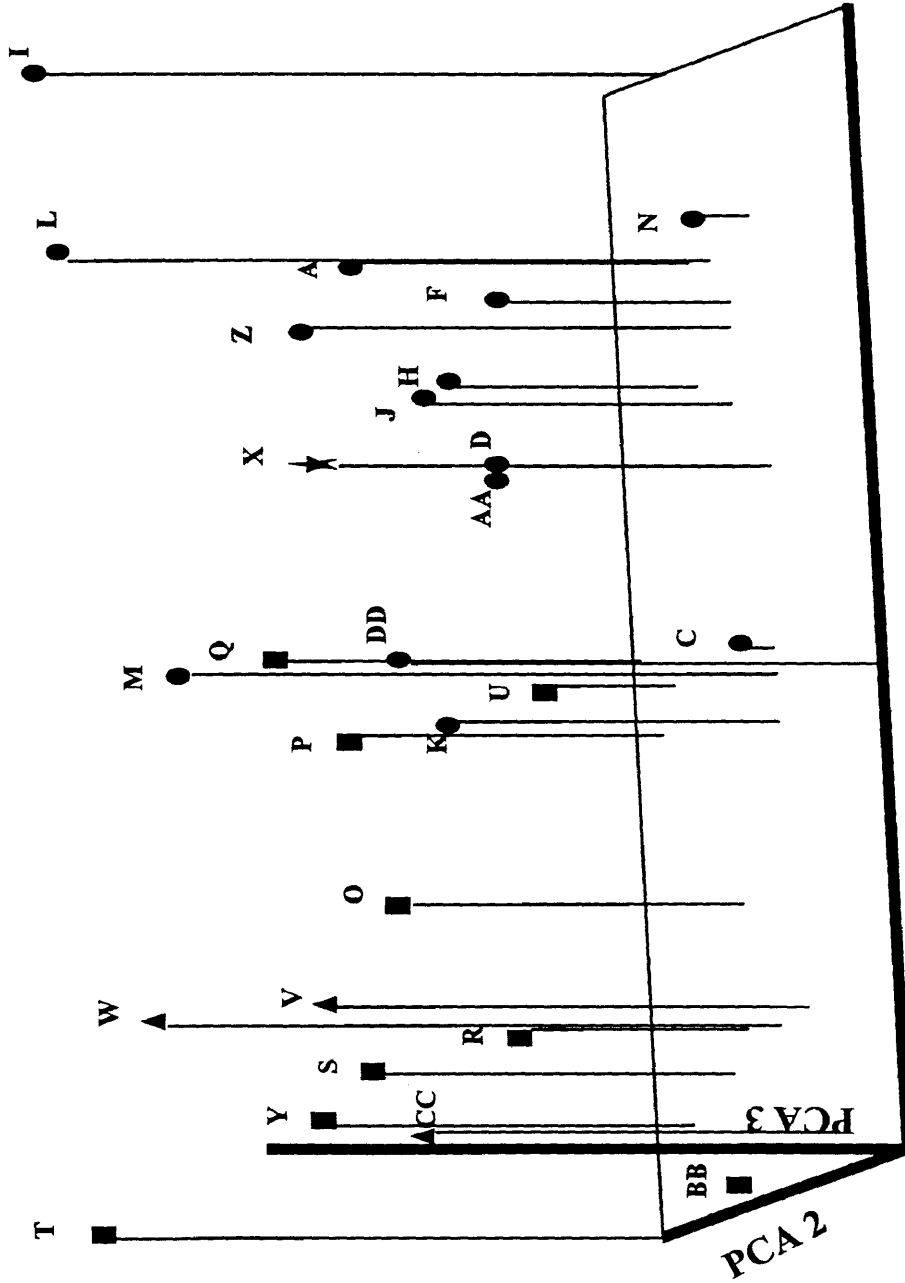
Figure 5. Plot of the first three principal component axes in relation to the distribution of populations based on population means of morphological characters. The first three axes account for 63.66%, 11.20%, and 7.85% of the variation, respectively. *Pubescens* populations are indicated by circles, *parviflorum* populations by squares, *makasin* populations by triangles, and the hybrid population by a star. Population letters are referenced in Table 1.

FIGURE 6. Cluster analysis resulting from Average Taxonomic Distance between populations. This analysis is based on morphology and UPGMA. Cophenetic correlation= 0.73. Population letters correspond to populations listed in Table 1. PARV= *parviflorum;* PUB= *pubescens*; MAK= *makasin*; BOTH= intermediate morphologies and varietally distinct morphologies present. Populations labeled as SYM were in sympatry with another variety (see Table 1).

Average Taxonomic Distance

```
|---------|---------|---------|---------|---------|---------|
1.750     1.500     1.250     1.000     0.750     0.500     0.250
```

```
                              .--------------------------PARV/PUB        X
                              |
                              |                    .-------PARV          P
                              |                    |
                              |              .--------L-------PARV        Q
                              |              |      |
                       .-----L---------L--------------PARV               U
                       |
                       |                    .-------------PUB            A
                       |                    |
                       |              .----L-------------PUB             F
                       |              |
                       |              |            .---------PUB/SYM Z
                       |              |            |
                       |              .L-------L----------PUB            L
                       |              |            |
                       |              |      .-------------PUB           D
                       |              |      |
                       |              |      |      .----------PUB/SYM        AA
                       |              |      |      |
                       |              |      |      |    .-------PUB           J
                       |              |      |      |    |
                       .-L--------------L----L--L--L--------PUB            H
                        |
                        |             .------------------------PUB           C
                        |             |
                        |             |    .-------------------PUB/SYM DD
                        |             |    |
                        |             |    |        .------------PUB           K
                        |             |    |        |
                 .---------L---------L--L---------L------------PUB            M
                 |
                 |       .-------------------------------------PUB            I
                 |       |
         .----------L----L-------------------------------------PUB            N
```

(CONTINUED)

```
|                                      .----------------------PARV          O
|                                      |
|                                      |        .-----PARV          S
|                                      |        |
|                                      .----------------L-----PARV/SYM      Y
|                                      |
|                                      |     .------------PARV          R
|                                      |     |
|                      .---L-------L--------------PARV/SYM      BB
|                      |
|                      |              .------------MAK          V
|                      |              |
|                      |           .--L------------MAK          W
|                      |           |
|           .-------L----------L---------------MAK/SYM     CC
|           |
L-------------------L---------------------------------PARV          T

|---------|---------|---------|---------|---------|---------|
1.750     1.500     1.250     1.000     0.750     0.500     0.250
```
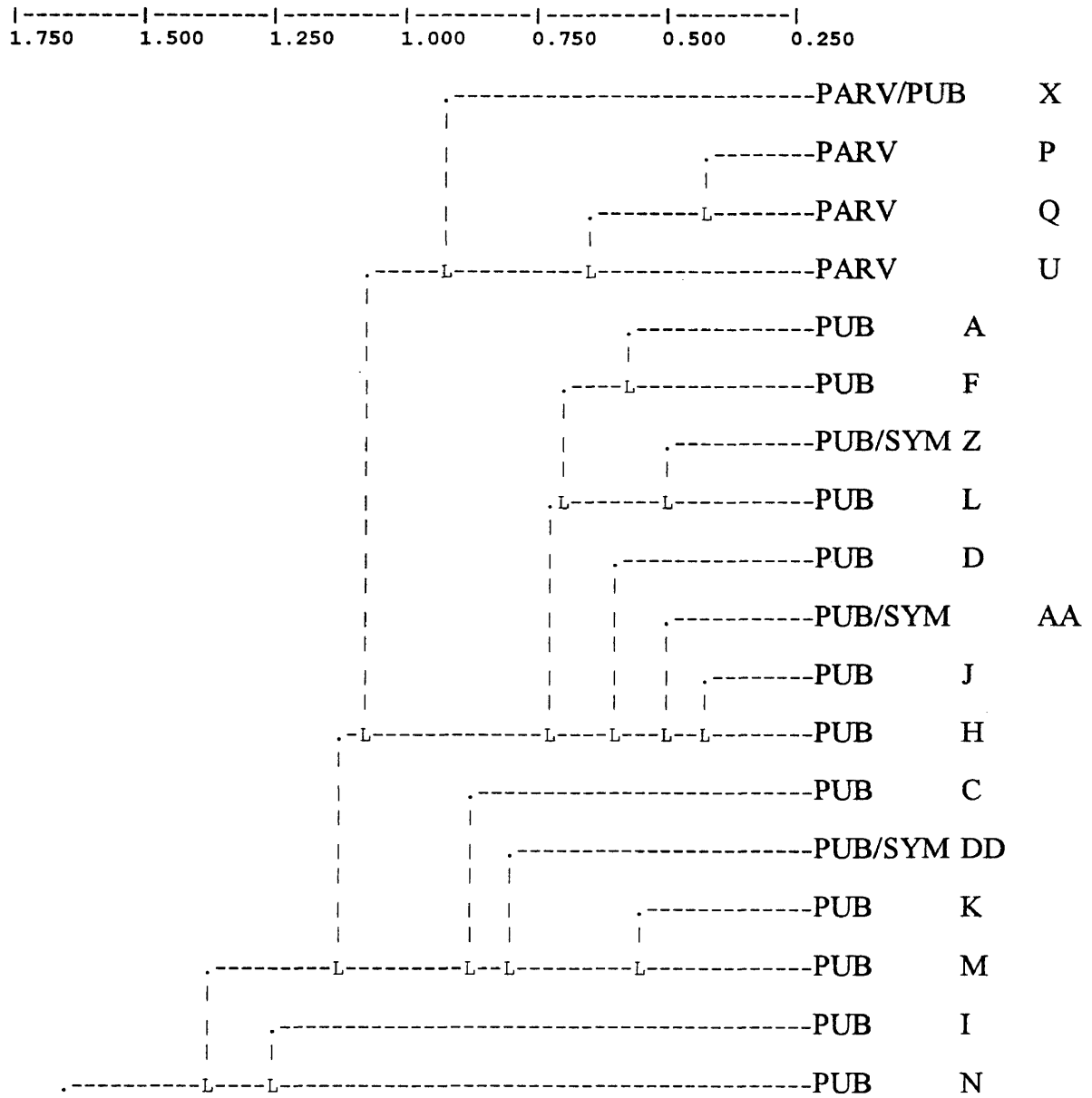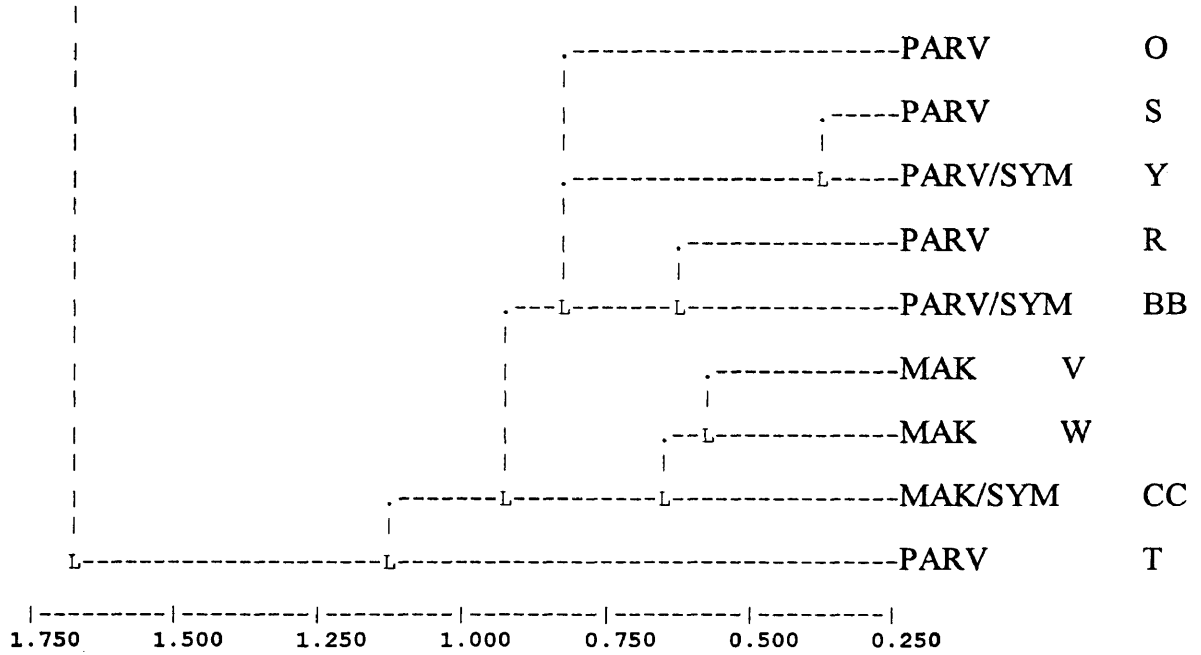
Average Taxonomic Distance

Figure 7. UPGMA phenogram based on Nei's (1978) Unbiased Genetic Identity. Cophenetic correlation= 0.82. Taxonomic status (PUB= *pubescens;* PARV= *parviflorum;* MAK= *makasin;* BOTH= intermediate morphologies and varietally distinct morphologies present), general geographic region (S= south; N= north), and population letters (Table 1) are given for each population. Populations labeled as SYM were in sympatry with a population of another variety.

Genetic Identity

```
+----+----+----+----+----+----+----+----+----+----+----+----+
.90       .92       .93       .95       .97       .98      1.00
```

```
                                                  *  PARV/PUB  S      X
                                                  *
                                              **** PUB         S      F
                                              *  *
                                              *  * PARV/SYM    S      BB
                                          *****
                                          *  *  * PUB         S      A
                                          *  ****
                                          *  ** * PUB         N      H
                                      ******  **
                                      *     *   * * PUB/SYM   N      DD
                                      *     *   ***
                                      *     *   * PUB         N      M
                                    **      *
                                    **  ******** PARV         S      U
                                    **
                                    **        *** PUB         S      B
                                    *************
                                    *         *** PUB         S      J
                                    *
                            ********* *       * PARV          S      O
                            *         *           **
                            *         *   ******** PUB         S      N
                            *         *     *      *
                            *         *  ****    ** PARV        S      P
                            *         *  *  *
                            *         *  *  ******** PUB        S      E
                            *         *  *
                            **    *****     * PARV            S      S
                            **        *  ********
                            **        *  *   * PUB            S      D
                            **        *  *
                            **        *********** PUB          S      G
                            **            *
                            ***        ******** PUB/SYM        S      Z
                            ***
                            ***        ************* PARV/SYM  S      Y
                            *************
                          ***** ************ PUB              N      I
                          *  **
                          *  ************************** PARV   N      T
```

(CONTINUED)

```
                    *******  *
                    *        *   ************************* PUB      N    K
                    *        *
                    *        *         ***************** PUB      S    C
        *******************      *************
                    *        *            **************** PUB      N    L
             *        *
                    *        *
    *******            *          ***************** PARV     S    Q
    *        *           ****************
    *        *              ***************** PARV     S    R
    *        *
    *        ********************************************************** PUB/SYM  S    AA
    *
    *                        ************** MAK      N    V
    *        *********************************
    *****************              ************** MAK/SYM  N    CC
             *
        ****************************************** MAK      N    W
+----+----+----+----+----+----+----+----+----+----+----+
.90       .92       .93       .95       .97       .98       1.00
```

Genetic Identity
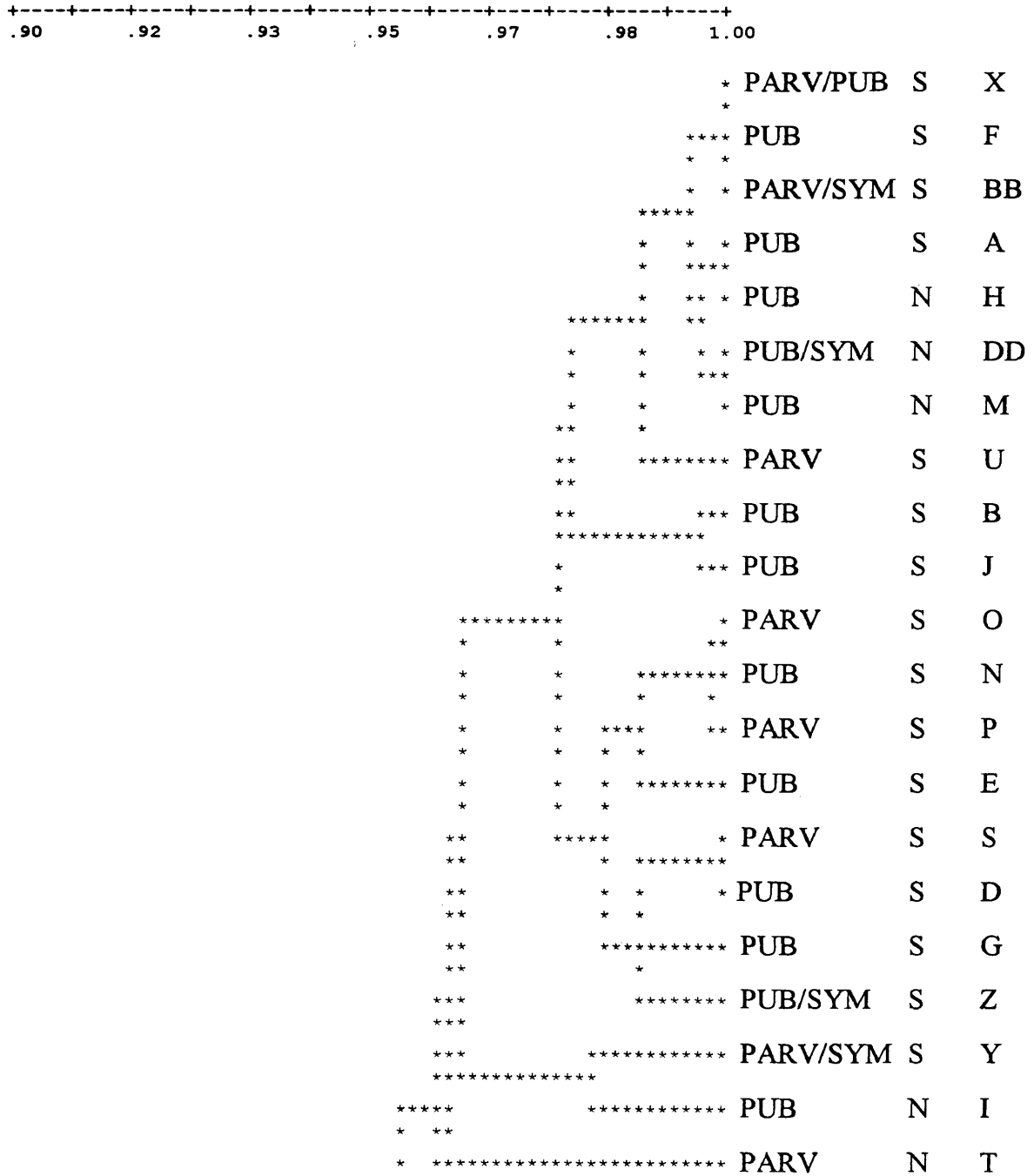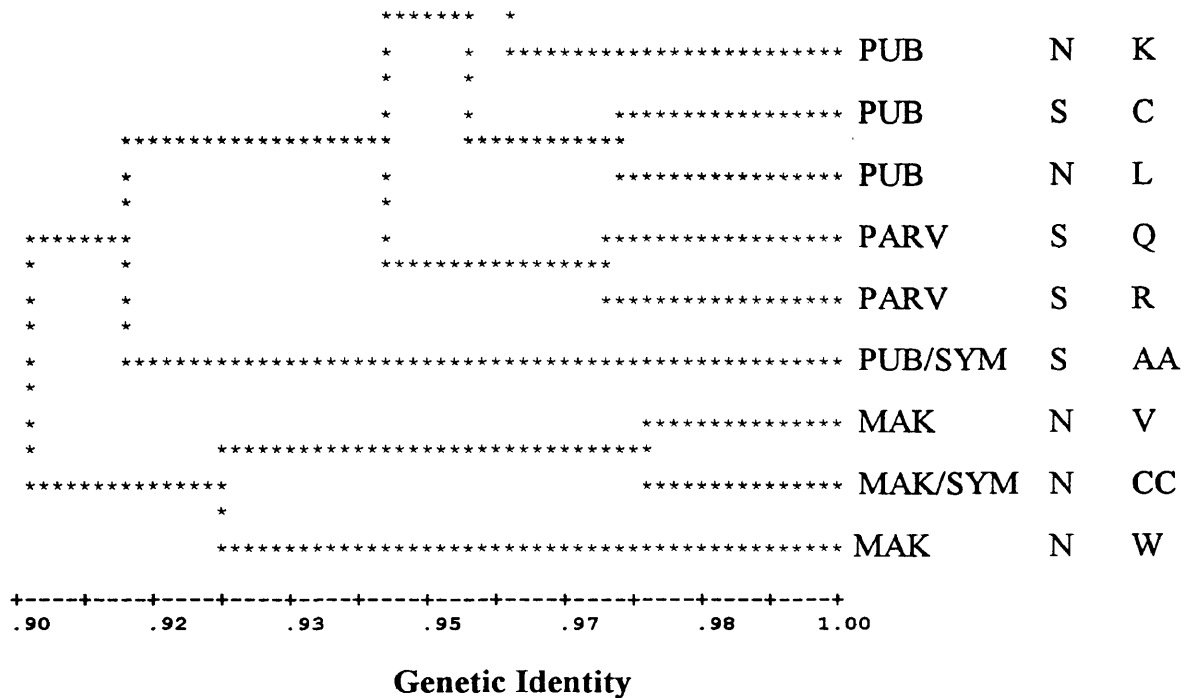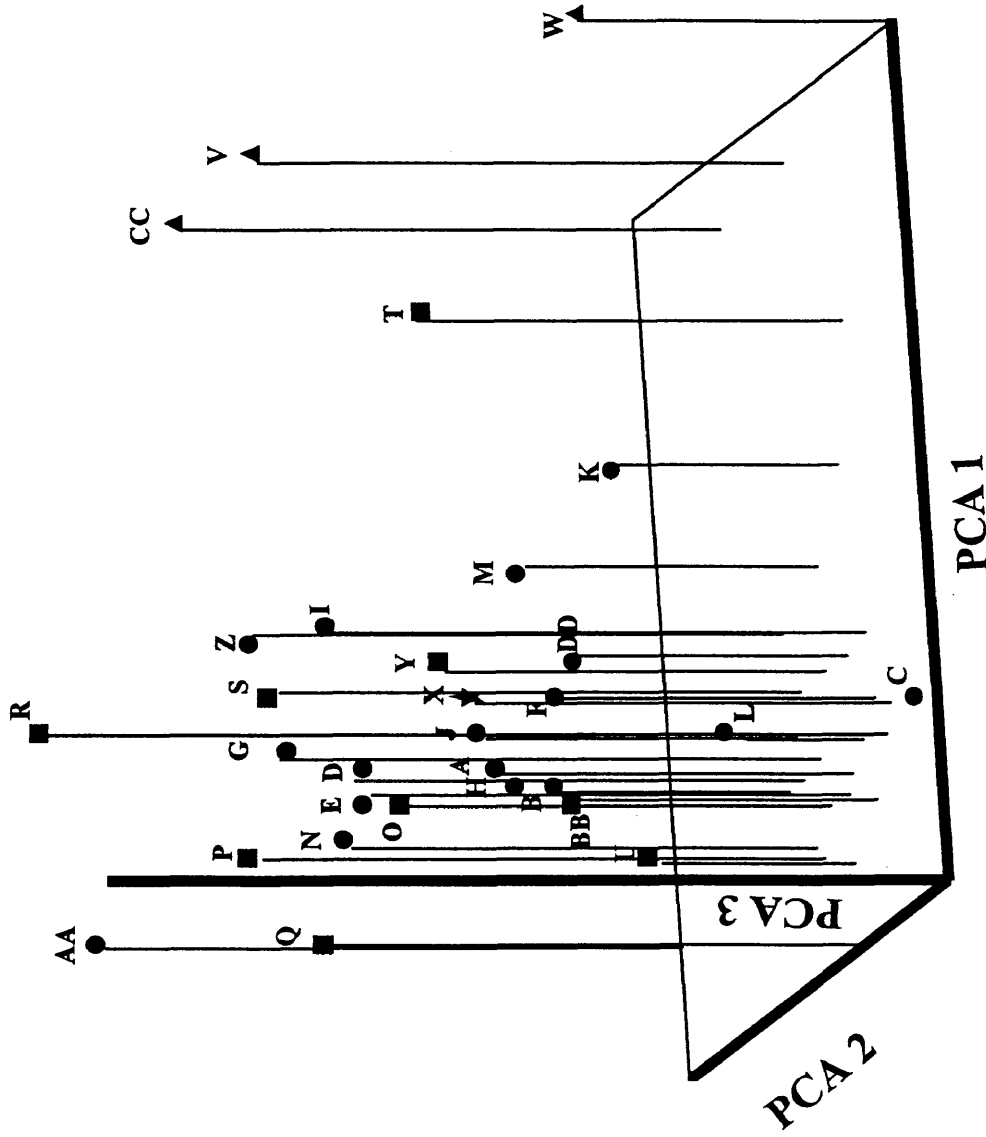
Figure 8. Plot of the first three principal component axes in relation to the distribution of populations based on allele frequencies. The first three axes account for 26.71%, 21.25%, and 12.80% of the variation, respectively. *Pubescens* populations are indicated by circles, *parviflorum* populations by squares, *makasin* populations by triangles, and the hybrid population by a star. Population letters are referenced in Table 1.

Figure 9. UPGMA cluster analysis based on Nei's (1978) Unbiased Genetic Identities for the populations in the present study combined with those of Case (1993). Cophenetic correlation= 0.89. Taxonomic status (PUB= *pubescens*; PARV= *parviflorum*; MAK= *makasin*; general geographic region (S= south, N= north), and population letters (Table 1) are given for each population. Taxa separated by a slash (/) indicate intermediate morphologies and varietally distinct morphologies present. Populations labeled as SYM were in sympatry with a population of another variety with no intermediate morphologies present. Populations from Case (1993) are indicated by an asterisk (*).

Genetic Identity

```
+----+----+----+----+----+----+----+----+----+----+----+
 .60      .67      .73      .80      .87      .93     1.00
```

*	PARV/PUB	S	X
**	PUB	S	F
**	PARV-SYM	S	BB
**	PUB	S	A
**	PUB	N	H
**	PUB-SYM	N	DD
**	PUB	N	M
***	PUB	N	FF*
***	PARV	S	O
***	PUB	N	II*
***	PUB	N	EE*
***	PUB	N	L
***	MAK/PUB	N	TT*
** *	PARV	S	P
****	PUB	S	D
****	PUB	S	N
****	PUB	N	KK*
* **	PARV	S	R
* **	PARV	S	S
** **	PUB	S	G

(CONTINUED)

```
* *
* * * * PARV        S    Q
* * * *
* *  * * PUB        S    E
       * *
* * * * * PUB       N    HH*
       *
*     * PUB         S    B
*    * *
*    * * PUB        S    J
  * * * * * *
*  *   * * PARV-SYM S    Y
*  *   * *
*  *    * PUB       N    I
*  *
*  *  * * * PARV    N    T
* *  * * *
* *  * * * * * PUB  N    GG*
* *    *
* *   * * * * MAK/PUB N  SS*
* *
* * * * *   * * * PUB-SYM S  Z
*   * * * * * *
*    *    * * * PUB  N    JJ*
*    *
*    *   * * * * PUB S    C
*   * * * * *
* *   * *  * * * * PARV S  U
* *    *
* *   * * * * * * PUB N   K
* *
* *        * MAK   N    V
* *       * * *
* * * * * * * * * * * MAK/PUB N PP*
* * * * * * *       *
*     *      * * * MAK/PUB N  QQ*
```

(CONTINUED)

```
              *       *
              *       *              ** MAK        N    W
              *       *            ***
              *       *           *  ** MAK        N    OO*
          ******      *    *****
              *       *     *   *  * ** MAK        N    MM*
              *       *   *****   ***
              *       *       *    ** MAK/PUB      N    RR*
      ********        *           *
          *       *   *   ******* MAK             N    LL*
          *       *   *
          *       * ***************** PUB-SYM      S    AA
          *       *
          *  ********************** MAK-SYM        N    CC
          *
      ****************************** MAK           N    NN*
  +----+----+----+----+----+----+----+----+----+----+----+
  .60      .67      .73      .80      .87      .93     1.00
```

Genetic Identity
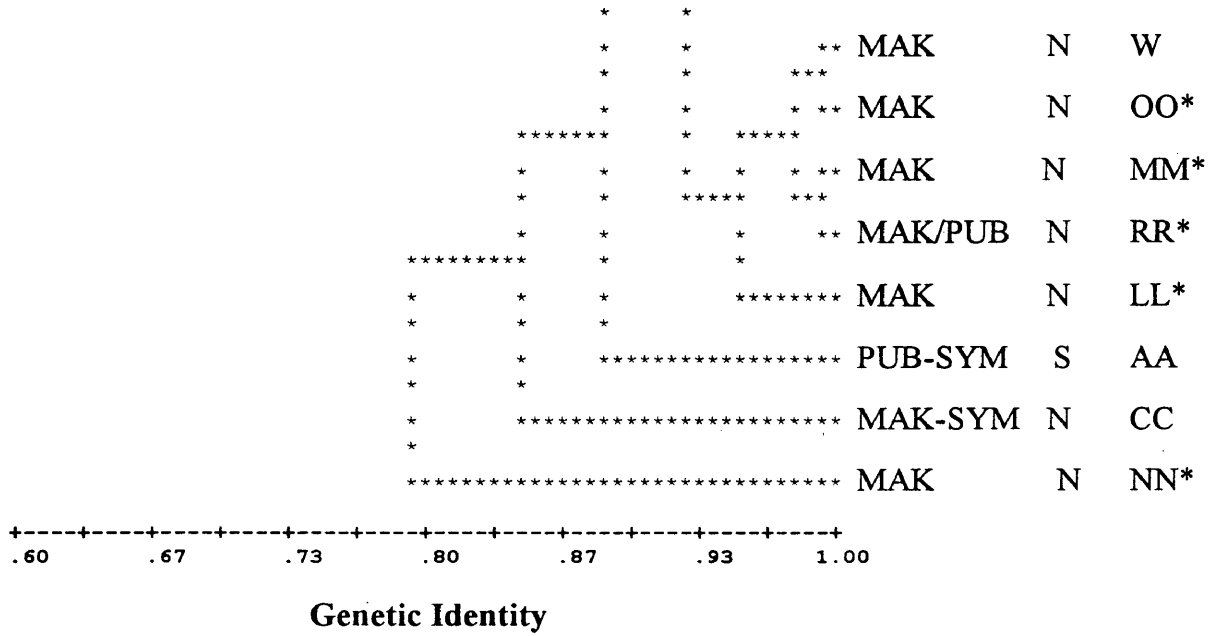
Appendix 1. Morphological measurements of individuals sampled. All measurements are in centimeters except the number of twists per petal. Voucher specimens are indicated by an *. Populations are referenced in Table 1.

Population (Variety and Individual)	Plant Height	No. Of Twists/ Petal	Staminode Length	Leaf Length	Leaf Width	Petal Length	Petal Width	Dorsal Sepal Length	Dorsal Sepal Width	Lateral Sepal Length	Lateral Sepal Width
A (PUB)											
1	51.00	3.00	1.50	17.25	8.50	8.00	0.75	6.50	2.75	5.25	2.00
2	48.75	3.00	1.25	16.25	7.25	8.00	0.75	6.00	2.00	5.50	2.00
3*	58.00	6.00	1.50	20.25	7.00	8.25	0.75	6.50	2.25	6.00	2.00
C (PUB)											
1	46.50	2.00	1.00	13.50	7.25	5.50	0.75	4.00	2.00	3.75	1.75
D (PUB)											
1	32.50	1.00	0.75	11.75	5.25	4.50	0.75	3.50	1.50	3.50	1.50
2	44.50	4.00	1.25	16.75	7.50	7.75	0.75	6.50	2.25	6.00	2.50
3	46.00	5.00	1.25	15.75	6.50	9.00	1.00	7.00	2.50	6.00	2.50
F (PUB)											
1	51.00	3.00	1.50	21.00	10.00	7.50	1.00	7.50	2.50	6.00	2.00
2	33.00	3.00	1.00	15.00	7.50	7.00	0.50	6.00	2.00	5.00	2.00
H (PUB)											
1*	50.50	3.00	1.00	16.00	8.75	7.00	0.75	5.75	2.50	4.50	1.75
2	50.00	4.00	1.00	16.50	8.50	7.00	0.50	6.00	2.00	5.00	2.00
3	52.50	3.00	1.25	15.00	9.50	7.00	0.75	6.25	2.25	5.00	2.50
4	49.00	4.00	1.00	15.50	8.75	7.00	0.50	6.00	2.00	5.00	2.00

Appendix 1, continued

Population (Variety and Individual)	Plant Height	No. of Twists/ Petal	Staminode Length	Leaf Length	Leaf Width	Petal Length	Petal Width	Dorsal Sepal Length	Dorsal Sepal Width	Lateral Sepal Length	Lateral Sepal Width
J (PUB)											
1	39.50	4.00	1.50	13.00	7.10	6.80	0.50	5.10	2.00	4.70	1.60
2	38.10	2.00	1.20	14.80	8.20	5.90	0.70	4.70	2.70	4.00	2.40
3	46.90	1.00	1.40	15.90	8.00	8.30	0.80	6.50	2.50	5.70	2.20
4	49.40	3.00	1.30	17.00	8.20	8.20	0.80	6.10	2.00	6.20	2.10
5	50.90	6.00	1.20	19.30	8.10	9.20	0.60	7.00	2.50	6.10	2.10
6	39.10	3.00	1.00	11.20	7.60	6.40	0.50	5.00	2.40	4.30	1.20
K (PUB)											
1*	57.80	3.00	1.40	18.50	6.40	6.00	0.90	4.90	2.70	4.40	2.40
2*	44.50	4.00	0.90	12.00	2.90	5.60	0.50	3.90	1.60	3.40	1.20
3*	33.75	4.00	1.20	11.00	3.90	5.50	0.50	3.80	1.80	3.50	1.40
4*	36.00	2.00	1.20	14.00	4.40	4.60	0.50	3.50	1.80	3.10	1.40
5*	50.25	2.00	1.00	13.00	4.70	4.40	0.60	3.50	1.70	3.10	1.50
6*	49.00	3.00	1.20	13.00	5.80	4.50	0.50	4.00	1.60	3.50	1.70
L (PUB)											
1*	49.00	4.00	1.30	14.00	7.00	7.50	0.50	5.50	2.00	5.00	1.50
2*	42.50	5.00	1.30	13.30	8.00	8.50	0.50	5.50	2.90	5.30	1.90
3*	52.50	7.00	1.70	15.30	8.40	10.50	0.90	7.50	2.50	5.00	2.50
M (PUB)											
1*	40.75	4.00	1.20	14.00	3.10	6.50	0.50	4.00	2.00	4.00	1.50

Appendix 1, continued

Population (Variety and Individual)	Plant Height	No. of Twists/ Petal	Staminode Length	Leaf Length	Leaf Width	Petal Length	Petal Width	Dorsal Sepal Length	Dorsal Sepal Width	Lateral Sepal Length	Lateral Sepal Width
N (PUB)											
1*	51.90	2.50	1.40	16.50	9.20	7.70	0.70	6.00	2.50	—	—
O (PARV)											
1*	39.00	4.00	1.00	16.00	6.00	4.50	0.50	4.00	1.50	3.00	1.00
2	30.50	4.00	0.75	13.00	4.50	4.50	0.50	4.00	1.50	3.75	1.00
3	33.00	4.00	1.00	11.50	5.00	5.50	0.50	4.00	1.50	4.00	1.00
4	37.00	5.00	1.25	11.00	5.75	5.00	0.75	4.00	2.00	3.00	1.75
5	52.50	3.00	1.25	14.00	7.50	5.00	1.00	4.50	2.00	3.50	1.50
P (PARV)											
1	47.00	4.00	1.00	15.00	6.00	6.00	0.50	4.50	1.50	5.00	1.00
2	40.00	5.00	1.00	13.00	6.00	5.75	0.50	4.50	1.75	3.75	1.00
3	46.00	3.00	1.00	19.75	6.25	6.50	0.50	6.00	1.50	5.75	1.50
4	52.00	3.00	1.25	19.00	9.50	6.50	0.50	6.00	2.00	5.00	1.50
5*	52.50	4.00	1.25	20.00	10.50	7.25	0.50	5.50	2.50	5.00	1.50
6	49.00	5.00	1.25	16.00	8.50	6.50	0.50	5.50	2.00	5.50	1.50
7	33.00	3.00	1.00	11.00	7.00	4.25	0.50	3.75	1.50	3.50	1.25
Q (PARV)											
1	55.00	4.00	1.00	17.75	11.50	5.50	0.75	5.00	2.00	4.00	1.00
2	47.00	3.00	1.00	15.00	9.25	5.50	0.50	4.50	1.75	4.00	1.25
3	51.00	4.00	1.00	15.75	7.50	6.25	0.50	5.00	2.00	4.50	1.50

Appendix 1, continued

Population (Variety and Individual)	Plant Height	No. of Twists/Petal	Staminode Length	Leaf Length	Leaf Width	Petal Length	Petal Width	Dorsal Sepal Length	Dorsal Sepal Width	Lateral Sepal Length	Lateral Sepal Width
4	44.00	6.00	1.25	15.50	7.50	6.75	0.50	5.25	1.75	4.75	1.75
5	48.50	5.00	1.25	15.25	9.50	6.00	0.50	4.75	2.00	4.00	1.75
6	50.00	6.00	1.25	19.50	9.75	6.00	0.50	5.00	2.25	4.00	1.75
7	53.75	7.00	1.00	18.00	7.50	6.50	0.50	5.50	2.00	5.00	1.50
8	52.50	4.00	1.25	15.25	7.50	5.25	0.50	5.25	2.25	4.50	2.00
9	47.25	4.00	1.00	16.00	7.50	5.00	0.50	5.00	1.75	4.50	1.25
10	61.00	7.00	1.25	19.50	9.00	6.50	0.25	5.50	1.50	5.00	1.25
11	49.75	4.00	0.75	13.50	11.25	6.00	0.50	5.50	2.25	4.25	1.75
12*	49.00	4.00	1.25	14.50	7.00	5.50	0.50	5.00	2.25	4.25	1.75
13	48.00	4.00	1.00	17.75	7.00	5.50	0.50	5.50	1.50	4.75	1.25
R (PARV)											
1*	38.75	2.00	1.00	13.00	6.00	5.00	0.50	4.00	1.50	4.25	1.50
2	45.00	1.00	0.75	13.50	6.75	4.75	0.50	4.50	1.75	4.00	1.75
3	46.00	4.00	1.25	13.50	6.50	5.25	0.50	4.50	1.50	4.50	1.50
4	42.75	0.50	1.25	13.50	6.25	3.75	0.50	3.50	1.50	4.00	1.50
5	26.00	2.00	0.75	9.75	2.75	5.00	0.50	3.50	1.50	3.50	1.00
6*	35.00	2.00	1.25	10.50	4.50	4.00	0.50	3.50	1.75	3.00	1.50
S (PARV)											
1	38.50	2.00	0.75	11.00	6.00	4.00	0.25	3.50	1.50	2.75	1.25
2	36.50	3.00	1.00	9.50	5.75	3.00	0.50	3.00	1.25	2.00	1.25

Appendix 1, continued

Population (Vareity and Individual)	Plant Height	No. of Twists/Petal	Staminode Length	Leaf Length	Leaf Width	Petal Length	Petal Width	Dorsal Sepal Length	Dorsal Sepal Width	Lateral Sepal Length	Lateral Sepal Width
3	46.00	5.00	1.00	9.50	10.00	5.50	0.50	4.50	2.00	4.00	0.75
T (PARV)											
1	41.50	6.00	0.75	15.75	5.00	5.00	0.25	3.75	1.25	3.50	1.00
2	40.00	4.00	0.75	13.50	3.00	4.50	0.25	3.50	1.50	3.00	1.25
U (PARV)											
1*	49.00	—	1.10	18.00	9.00	4.40	0.60	4.10	2.00	—	—
V (MAK)											
1*	35.50	4.00	1.30	11.90	4.70	4.50	0.40	3.50	1.60	3.70	1.30
2*	29.10	4.00	1.00	9.50	2.50	4.50	0.40	3.20	1.20	2.60	1.10
3*	34.50	3.00	1.00	11.30	3.90	3.80	0.50	3.00	1.40	2.80	1.20
W (MAK)											
1*	35.50	4.00	1.20	11.00	3.80	4.50	0.40	3.60	1.40	3.00	0.90
X (MIXED)											
1 (PARV)*	45.00	4.00	1.00	16.50	8.00	5.00	0.50	4.00	2.00	4.00	1.50
2 (PUB)	60.50	6.00	1.00	18.00	9.00	7.00	0.75	6.00	2.00	5.00	1.75
3 (PUB)*	59.00	5.00	1.25	16.00	9.00	6.00	0.50	5.00	2.50	3.75	2.00
4 (PARV)	48.00	6.00	1.25	16.00	8.50	6.50	0.50	5.00	2.25	4.50	1.75
Y (SYM PARV)											
1	32.00	3.00	0.75	4.25	9.50	3.25	0.25	2.50	1.25	2.00	1.00

Appendix 1, continued

Population (Variety and Individual)	Plant Height	No. of Twists/ Petal	Staminode Length	Leaf Length	Leaf Width	Petal Length	Petal Width	Dorsal Sepal Length	Dorsal Sepal Width	Lateral Sepal Length	Lateral Sepal Width
2	55.50	4.00	1.00	15.00	9.00	5.00	0.50	4.00	1.75	3.50	1.00
3	43.00	3.00	0.75	11.75	6.50	5.00	0.25	4.50	1.75	3.50	1.00
Z (SYM PUB)											
1	42.50	4.00	1.25	13.50	7.50	7.50	0.75	5.50	2.25	5.00	2.00
2	52.00	5.00	1.25	13.00	10.00	8.50	0.75	7.00	2.50	5.50	2.50
3	45.00	4.00	1.25	13.25	8.25	7.00	0.75	5.75	2.00	5.00	2.00
AA (SYM PUB)											
1	33.50	2.00	1.25	15.00	5.50	7.00	0.50	5.75	2.00	4.00	1.75
2	47.00	3.00	1.00	15.75	6.75	8.00	0.75	6.50	2.50	5.00	2.00
BB (SYM PARV)											
1*	38.00	1.00	0.75	13.00	5.50	4.25	0.50	3.50	1.50	3.00	1.00
CC (SYM MAK)											
1*	29.30	2.00	0.80	10.00	1.90	3.30	0.40	2.70	1.30	2.50	1.10
2*	29.00	3.00	0.90	9.60	3.50	3.90	0.50	2.80	1.40	2.40	1.30
3*	29.80	3.00	1.10	9.50	2.50	4.50	0.50	3.70	1.50	3.50	1.50

Appendix 1, continued

Population (Variety and Individual)	Plant Height	No. of Twists/ Petal	Staminode Length	Leaf Length	Leaf Width	Petal Length	Petal Width	Dorsal Sepal Length	Dorsal Sepal Width	Lateral Sepal Length	Lateral Sepal Width
DD (SYM PUB)											
1*	29.00	3.00	1.10	9.70	2.90	5.10	0.50	4.00	1.70	3.90	1.70
2*	27.80	4.00	1.30	9.00	2.80	6.60	0.60	4.40	2.00	3.90	1.50
3*	31.00	2.00	1.30	12.00	4.00	5.50	0.90	4.00	2.20	3.50	2.10

Appendix 1, continued

Population (Variety and Individual)	Slipper Length	Slipper Width	Orifice Length	Orifice Width
A (PUB)				
1	4.25	2.25	0.75	1.00
2	3.50	2.00	0.75	0.75
3	4.00	2.25	1.25	0.75
C (PUB)				
1	3.25	1.75	1.00	1.00
D (PUB)				
1	2.50	1.50	0.50	0.50
2	4.00	2.25	1.00	1.00
3	4.00	2.00	1.00	1.25
F (PUB)				
1	4.50	3.00	1.00	1.00
2	3.50	2.00	0.75	0.75
H (PUB)				
1	3.75	1.75	0.75	1.00
2	4.00	2.50	1.00	1.25
3	4.00	2.00	1.00	1.50
4	3.75	2.00	0.75	1.00
I (PUB)				
1	5.00	2.25	1.00	1.25

Appendix 1, continued

Population (Variety and Individual)	Slipper Length	Slipper Width	Orifice Length	Orifice Width
2	4.50	2.25	1.00	1.25
J (PUB)				
1	4.00	1.20	0.60	1.10
2	3.90	2.00	0.90	1.00
3	3.70	2.40	1.10	1.10
4	4.30	2.10	1.10	1.10
5	4.20	2.00	0.40	0.90
6	3.30	1.90	0.90	1.00
K (PUB)				
1	4.00	2.90	1.00	1.00
2	3.00	2.00	1.00	1.10
3	3.40	2.20	0.80	0.90
4	3.30	1.90	1.00	0.80
5	2.60	2.00	0.60	0.70
6	3.00	2.00	0.70	0.70
L (PUB)				
1	3.40	2.20	0.60	1.10
2	3.50	2.60	1.30	1.20
3	5.80	2.60	0.70	0.70

Appendix 1, continued

Population (Variety and Individual)	Slipper Length	Slipper Width	Orifice Length	Orifice Width
M (PUB)				
1	3.90	2.10	0.70	1.00
N (PUB)				
1	3.30	2.50	1.50	1.50
O (PARV)				
1	2.00	1.25	0.50	0.75
2	2.00	1.00	0.75	0.75
3	2.50	1.50	0.75	1.00
4	2.00	1.25	0.75	0.50
5	3.00	1.75	0.75	1.00
P (PARV)				
1	2.25	1.00	0.75	0.75
2	2.50	1.50	1.00	1.00
3	2.75	1.25	0.75	1.00
4	3.00	1.75	0.50	1.00
5	3.00	1.75	0.50	1.00
6	2.25	1.00	0.50	1.00
7	2.00	1.00	0.75	1.00
Q (PARV)				
1	2.50	1.75	0.75	1.00

Appendix 1, continued

Population (Variety and Individual)	Slipper Length	Slipper Width	Orifice Length	Orifice Width
2	2.25	1.75	0.75	1.00
3	2.75	1.50	0.75	0.75
4	2.75	1.50	1.25	1.00
5	3.00	1.50	1.25	1.00
6	2.75	1.25	0.50	0.75
7	3.00	1.75	0.75	0.75
8	3.25	1.75	1.00	0.75
9	3.00	1.00	0.75	0.75
10	2.75	1.25	0.50	0.75
11	3.50	2.25	1.25	1.00
12	3.50	2.00	0.50	1.00
13	3.00	1.00	0.50	0.75
R (PARV)				
1	2.25	1.50	0.50	0.75
2	1.50	0.75	0.25	0.50
3	2.50	1.75	0.50	0.75
4	2.25	1.00	0.50	1.00
5	2.50	1.25	0.50	0.50
6	1.25	0.50	0.25	0.25

Appendix 1, continued

Population (Variety and Individual)	Slipper Length	Slipper Width	Orifice Length	Orifice Width
S (PARV)				
1	2.00	1.25	0.50	0.50
2	2.00	1.00	0.75	0.50
3	3.00	1.75	0.75	1.00
T (PARV)				
1	1.75	1.00	0.50	0.25
2	2.00	1.00	0.50	0.75
U (PARV)				
1	2.80	1.60	1.00	0.70
V (MAK)				
1	2.70	1.60	1.00	0.80
2	2.50	1.50	1.70	0.60
3	2.30	1.50	0.60	0.80
W (MAK)				
1	2.80	1.70	0.70	0.70
X (MIXED)				
1 (PARV)	2.25	1.00	0.75	0.75
2 (PUB)	3.75	2.00	1.25	1.00
3 (PUB)	3.50	2.00	1.00	1.25
4 (PARV)	3.00	2.00	1.25	1.50

Appendix 1, continued

Population (Variety and Individual)	Slipper Length	Slipper Width	Orifice Length	Orifice Width
Y (SYM PARV)				
1	2.00	1.25	0.25	0.50
2	2.75	1.50	0.50	0.50
3	2.25	1.25	0.75	0.50
Z (SYM PUB)				
1	4.00	2.25	1.00	1.00
2	4.00	2.25	0.75	1.00
3	4.00	2.50	0.75	1.00
AA (SYM PUB)				
1	3.50	2.00	0.75	1.00
2	3.75	2.25	1.00	1.25
BB (SYM PARV)				
1	1.75	1.00	0.50	0.75
CC (SYM MAK)				
1	2.20	1.20	0.70	0.60
2	2.50	1.50	0.70	0.80

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Appendix 1, continued

Population (Variety and Individual)	Slipper Length	Slipper Width	Orifice Length	Orifice Width
3	2.60	1.40	0.80	0.80
DD (SYM PUB)				
1	3.50	2.10	0.90	0.80
2	3.50	2.20	0.90	0.90
3	3.90	2.90	1.10	1.30

Appendix 2. Average Taxonomic Distance (below diagonal) and Nei's Genetic Identity (above diagonal) for all populations. Missing values represent populations for which data were not available.

POP	A	B	C	D	E	F	G	H	I	J	K	L
A	***	0.973	0.960	0.992	0.972	1.000	0.969	1.000	0.958	0.980	0.973	0.974
B	—	***	0.953	0.989	0.972	0.980	0.948	0.974	0.974	0.996	0.962	0.962
C	1.281	—	***	0.933	0.915	0.968	0.907	0.966	0.932	0.947	0.944	0.973
D	0.849	—	0.805	***	0.979	0.999	0.992	0.990	0.999	0.993	0.972	0.971
E	—	—	—	—	***	0.970	0.979	0.974	0.931	0.995	0.944	0.965
F	0.599	—	1.169	0.694	—	***	0.969	1.000	0.974	0.982	0.978	0.980
G	—	—	—	—	—	—	***	0.973	0.979	0.978	0.936	0.967
H	0.718	—	0.904	0.703	—	0.685	—	***	0.962	0.983	0.968	0.980
I	0.989	—	1.835	1.428	—	1.184	—	1.047	***	0.977	0.946	0.946
J	0.638	—	0.896	0.552	—	0.565	—	0.434	1.070	***	0.965	0.979
K	1.281	—	0.641	0.940	—	1.226	—	1.018	1.835	0.953	***	0.952
L	0.658	—	1.429	1.007	—	0.877	—	0.804	0.751	0.720	1.311	***
M	1.279	—	1.028	1.011	—	1.265	—	1.074	1.690	0.930	0.573	1.154
N	1.178	—	1.390	1.414	—	1.209	—	1.069	1.252	1.160	1.578	1.317
O	1.595	—	0.919	1.120	—	1.534	—	1.343	2.143	1.245	0.747	1.618
P	1.216	—	0.984	1.020	—	1.219	—	0.942	1.684	0.915	0.894	1.323
Q	1.064	—	1.002	1.021	—	1.149	—	0.807	1.522	0.885	0.897	1.140
R	1.884	—	1.161	1.409	—	1.779	—	1.614	2.400	1.487	1.015	1.976
S	2.005	—	1.184	1.572	—	1.915	—	1.644	2.409	1.598	1.007	1.959
T	2.336	—	1.815	1.987	—	2.315	—	2.076	2.768	2.069	1.518	2.342
U	1.218	—	0.800	1.089	—	1.218	—	1.041	1.790	1.076	0.926	1.473
V	2.001	—	1.242	1.569	—	1.924	—	1.741	2.414	1.635	0.943	1.943
W	1.946	—	1.355	1.594	—	1.890	—	1.719	2.364	1.596	0.913	1.846

Appendix 2, continued

POP	A	B	C	D	E	F	G	H	I	J	K	L
X	1.147	—	1.140	1.175	—	1.273	—	0.751	1.349	0.986	1.182	1.112
Y	2.113	—	1.396	1.746	—	2.033	—	1.765	2.504	1.741	1.220	2.077
Z	0.658	—	1.087	0.615	—	0.661	—	0.544	0.959	0.499	1.118	0.519
AA	0.922	—	0.821	0.583	—	0.690	—	0.600	1.350	0.445	0.851	0.979
BB	2.262	—	1.315	1.745	—	2.105	—	1.907	2.795	1.843	1.295	2.374
CC	2.252	—	1.359	1.679	—	2.110	—	1.936	2.690	1.813	1.113	2.209
DD	1.603	—	1.024	1.053	—	1.407	—	1.364	1.965	1.174	0.803	1.461

Appendix 2, continued

POP	M	N	O	P	Q	R	S	T	U	V	W	X
A	0.996	0.990	0.996	0.987	0.968	0.953	0.976	0.964	0.993	0.914	0.893	0.989
B	0.971	0.981	0.973	0.964	0.934	0.907	0.969	0.939	0.974	0.889	0.873	0.961
C	0.951	0.936	0.944	0.921	0.920	0.890	0.917	0.921	0.968	0.856	0.895	0.957
D	0.984	0.995	0.994	0.991	0.940	0.951	1.000	0.977	0.967	0.922	0.903	0.986
E	0.972	0.991	0.994	0.983	0.957	0.935	0.982	0.951	0.966	0.927	0.859	0.955
F	0.998	0.983	0.994	0.979	0.948	0.949	0.987	0.973	0.988	0.910	0.920	1.000
G	0.958	0.986	0.986	0.989	0.953	0.967	0.986	0.963	0.939	0.922	0.881	0.970
H	0.991	0.990	0.995	0.989	0.971	0.956	0.975	0.958	0.991	0.907	0.889	0.987
I	0.942	0.974	0.960	0.973	0.929	0.964	0.974	0.967	0.922	0.894	0.935	0.987
J	0.978	0.996	0.991	0.984	0.958	0.935	0.982	0.958	0.976	0.920	0.885	0.969
K	0.979	0.958	0.959	0.945	0.913	0.904	0.951	0.950	0.962	0.949	0.944	0.957
L	0.970	0.965	0.974	0.955	0.932	0.912	0.960	0.951	0.968	0.892	0.887	0.967
M	***	0.975	0.985	0.970	0.942	.931	0.973	0.974	.982	0.933	0.911	0.978
N	1.738	***	1.000	1.000	0.981	0.965	0.984	0.954	0.979	0.919	0.873	0.977
O	0.925	1.982	***	0.997	0.977	0.966	0.988	0.968	0.985	0.924	0.885	0.984
P	0.947	1.681	0.743	***	0.986	0.980	0.981	0.950	0.969	0.911	0.865	0.977
Q	0.989	1.590	0.900	0.435	***	0.971	0.935	0.903	0.964	0.872	0.816	0.951
R	1.213	2.375	0.728	0.987	1.250	***	0.954	0.933	0.921	0.898	0.865	0.958
S	1.229	2.277	0.673	1.052	1.167	0.702	***	0.975	0.951	0.931	0.902	0.975
T	1.553	2.876	1.142	1.324	1.442	1.134	0.998	***	0.932	0.957	0.956	0.966
U	1.292	1.601	0.997	0.776	0.560	1.306	1.260	1.683	***	0.884	0.863	0.968
V	1.103	2.167	0.802	1.261	1.356	1.059	0.792	1.203	1.411	***	0.942	0.904
W	0.947	2.247	0.738	1.152	1.279	0.924	0.693	1.065	1.410	0.557	***	0.915
X	1.209	1.186	1.342	0.971	0.779	1.786	1.658	1.988	1.042	1.685	1.713	***

Appendix 2, continued

POP	M	N	O	P	Q	R	S	T	U	V	W	X
Y	1.411	2.482	0.928	1.151	1.242	0.807	0.373	0.954	1.385	1.105	0.921	1.815
Z	1.112	1.243	1.404	1.176	1.032	1.729	1.754	2.223	1.269	1.800	1.753	1.056
AA	0.851	1.200	1.189	0.963	1.012	1.413	1.534	1.989	1.187	1.510	1.509	1.082
BB	1.535	2.515	0.981	1.279	1.546	0.624	0.808	1.192	1.390	1.192	1.159	1.997
CC	1.246	2.525	0.872	1.423	1.607	0.832	0.755	1.164	1.692	0.602	0.688	1.982
DD	0.818	1.798	1.088	1.402	1.468	1.367	1.356	1.910	1.618	1.038	1.126	1.580

Appendix 2, continued

POP	Y	Z	AA	BB	CC	DD
A	0.948	0.950	0.916	1.000	0.899	0.999
B	0.970	0.954	0.945	0.998	0.890	0.977
C	0.947	0.886	0.825	0.999	0.833	0.966
D	0.972	0.988	0.969	1.000	0.914	0.984
E	0.954	0.981	0.934	0.996	0.964	0.975
F	0.954	0.964	0.906	1.000	0.903	1.000
G	0.957	0.987	0.932	0.999	0.921	0.963
H	0.953	0.951	0.919	1.000	0.900	0.998
I	0.982	0.980	0.923	0.996	0.879	0.957
J	0.974	0.977	0.945	1.000	0.922	0.986
K	0.934	0.931	0.897	0.994	0.920	0.983
L	0.951	0.949	0.883	1.000	0.881	0.978
M	0.937	0.951	0.911	1.000	0.927	1.000
N	0.968	0.972	0.945	1.000	0.914	0.985
O	0.961	0.974	0.926	1.000	0.921	0.992
P	0.956	0.968	0.942	1.000	0.910	0.979
Q	0.930	0.915	0.890	0.997	0.867	0.962
R	0.934	0.937	0.885	0.981	0.890	0.944
S	0.976	0.988	0.945	0.998	0.937	0.971
T	0.941	0.973	0.881	0.983	0.940	0.965
U	0.939	0.921	0.896	1.000	0.872	0.993
V	0.907	0.929	0.849	0.926	0.977	0.925
W	0.907	0.899	0.770	0.919	0.909	0.912
X	0.954	0.959	0.888	1.000	0.891	0.985

Appendix 2, continued

POP	Y	Z	AA	BB	CC	DD
Y	***	0.954	0.908	0.984	0.911	0.949
Z	1.890	***	0.924	0.979	0.940	0.952
AA	1.706	0.754	***	0.925	0.867	0.906
BB	0.887	2.109	1.682	***	0.913	1.000
CC	1.027	1.997	1.642	0.881	***	0.915
DD	1.636	1.260	0.987	1.623	1.129	***

Appendix 3. Allele frequencies across all populations. Population letters are referenced in Table 1.

Locus & Allele	A	B	C	D	E	F	G	H	I
PGM-1									
a	—	—	—	—	—	—	—	—	—
b	—	—	—	—	—	—	—	—	—
c	1.000	1.000	0.667	1.000	0.938	1.000	0.762	0.897	1.000
d	—	—		—		—	—	—	—
e	—	—	0.333	—	0.062	—	0.238	0.103	—
PGM-2									
a	—	—	—		—		—	—	—
b	0.192	0.475	0.556	—	0.438	—	0.026	0.207	—
c	0.808	0.525	0.444	1.000	0.562	0.667	0.974	0.793	1.000
d	—	—	—	—	—	0.333	—	—	—
IDH-2									
a	0.923	0.950	1.000	1.000	1.000	1.000	1.000	1.000	1.000
b	0.077	0.050	—	—	—	—	—	—	—
c	—	—	—	—	—	—	—	—	—
SKD-1									
a	0.115	0.325	0.333	0.333	0.312	0.333	0.435	0.138	0.750
b	0.885	0.675	0.667	0.667	0.688	0.667	0.565	0.862	0.250
c	—	—	—	—	—	—	—	—	—
PGD-1									
a	—	0.025		—	—	—	—	—	—
b	1.000	0.800	1.000	1.000	0.812	0.833	1.000	0.897	1.000
c	—	0.050	—	—	—	0.167	—	0.017	—
d	—	0.125	—	—	0.188	—	—	0.086	—
PGI-1									
a	0.154	0.059	0.250	—	—	—	0.217	0.214	0.500
b	0.846	0.941	0.750	1.000	1.000	0.833	0.783	0.786	0.500
c	—	—	—	—	—	0.167	—	—	—
d	—	—	—	—	—	—	—	—	—
SOD-1									
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADH-1									
a	—	—	—	—	—	—	—	—	—
b	—	0.447	0.056	0.333	0.062	—	0.125	0.019	0.500

Appendix 3, continued

c	1.000	0.553	0.944	0.667	0.938	1.000	0.875	0.981	0.500
GDH-1									
a	—	—	—	—	—	—	—	—	—
b	—	—	—	—	—	—	—	—	—
c	1.000	1.000	0.722	1.000	1.000	1.000	1.000	0.966	1.000
d	—	—	0.278	—	—	—	—	0.034	—
e	—	—	—	—	—	—	—	—	—
f	—	—	—	—	—	—	—	—	—
GOT-1									
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GOT-2									
a	—	—	—	—	—	—	0.043	—	—
b	0.462	0.475	0.889	0.333	—	0.500	0.022	0.466	0.500
c	0.038	—	—	—	—	0.167	—	—	—
d	0.500	0.525	0.111	0.667	1.000	0.333	0.935	0.534	0.500
TPI-1									
a	—	0.050	—	—	—	—	0.043	0.034	0.250
b	1.000	0.950	1.000	1.000	1.000	1.000	0.957	0.966	0.750
TPI-2									
a	0.962	1.000	1.000	1.000	0.938	1.000	0.957	1.000	1.000
b	0.038	—	—	—	0.062	—	0.043	—	—

Appendix 3, continued

Locus & Allele	POPULATION								
	J	K	L	M	N	O	P	Q	R
PGM-1									
a	—	—	—	0.050	—	—	—	—	—
b	—	—	—	—	—	—	—	0.105	—
c	0.895	0.907	0.525	0.925	1.000	1.000	1.000	0.895	1.000
d	—	0.093	—	0.025	—	—	—	—	—
e	0.105	—	0.475	—	—	—	—	—	—
PGM-2									
a	—	—	—	—	—	—	—	—	—
b	0.421	0.241	0.350	0.225	0.300	0.269	0.156	0.421	—
c	0.553	0.759	0.650	0.775	0.700	0.731	0.844	0.579	1.000
d	0.026	—	—	—	—	—	—	—	—
IDH-2									
a	0.974	0.962	1.000	0.775	1.000	1.000	1.000	1.000	1.000
b	0.026	0.019	—	0.225	—	—	—	—	—
c	—	0.019	—	—	—	—	—	—	—
SKD-1									
a	0.342	0.259	0.350	0.200	0.225	0.231	0.188	0.026	0.324
b	0.658	0.741	0.650	0.800	0.775	0.769	0.812	0.974	0.676
c	—	—	—	—	—	—	—	—	—
PGD-1									
a	—	—	—	—	—	—	—	—	—
b	0.868	0.833	1.000	0.775	1.000	1.000	0.969	1.000	1.000
c	—	0.167	—	—	—	—	—	—	—
d	0.132	—	—	0.225	—	—	0.031	—	—
PGI-1									
a	0.106	—	0.025	—	0.250	0.154	0.344	0.632	0.647
b	0.868	0.889	0.975	0.925	0.750	0.846	0.625	0.368	0.353
c	—	—	—	—	—	—	—	—	—
d	0.132	0.111	—	0.075	—	—	0.031	—	—
SOD-1									
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADH-1									
a	—	0.037	—	—	—	—	—	—	—
b	0.289	0.167	0.025	—	0.194	—	0.125	—	—
c	0.711	0.796	0.975	1.000	0.806	1.000	0.875	1.000	1.000

Appendix 3, continued

GDH-1

a	—	—	—	—	—	—	—	—	—
b	—	0.037	—	—	—	—	—	—	—
c	1.000	0.852	1.000	0.925	1.000	0.962	1.000	0.974	0.676
d	—	0.037	—	0.050	—	0.038	—	—	—
e	—	0.074	—	0.025	—	—	—	0.026	0.294
f	—	—	—	—	—	—	—	—	0.030

GOT-1

a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

GOT-2

a	—	—	—	0.075	—	—	—	—	—
b	0.263	0.611	0.475	0.425	0.175	0.192	0.125	0.105	—
c	—	—	—	0.075	—	0.038	—	0.079	0.235
d	0.737	0.389	0.525	0.425	0.825	0.770	0.875	0.816	0.765

TPI-1

a	0.158	0.130	0.150	0.075	—	—	—	—	—
b	0.842	0.870	0.850	0.925	1.000	1.000	1.000	1.000	1.000

TPI-2

a	0.921	0.500	1.000	0.850	0.950	1.000	1.000	1.000	1.000
b	0.079	0.500	—	0.150	0.050	—	—	—	—

Appendix 3, continued

Locus & Allele	POPULATION								
	S	T	U	V	W	X	Y	Z	AA
PGM-1									
a	—	—	—	—	—	—	—	—	—
b	—	—	—	0.019	—	—	—	—	—
c	0.975	1.000	1.000	0.981	1.000	0.977	0.850	0.974	1.000
d	—	—	—	—	—	—	—	0.026	—
e	0.025	—	—	—	—	0.023	0.150	—	—
PGM-2									
a	0.075	—	—	—	—	0.250	—	—	—
b	0.050	0.050	0.487	0.173	0.075	—	0.400	0.105	—
c	0.875	0.950	0.513	0.827	0.925	0.750	0.600	0.895	1.000
d	—	—	—	—	—	—	—	—	—
IDH-2									
a	1.000	0.750	1.000	0.788	0.900	0.909	1.000	1.000	1.000
b	—	0.250	—	0.212	0.075	—	—	—	—
c	—	—	—	—	0.025	0.091	—	—	—
SKD-1									
a	0.425	0.550	—	0.481	0.825	0.432	0.550	0.684	—
b	0.575	0.450	1.000	0.500	0.175	0.568	0.450	0.316	1.000
c	—	—	—	0.019	—	—	—	—	—
PGD-1									
a	—	—	—	—	—	—	—	—	—
b	0.925	1.000	1.000	0.865	0.925	1.000	1.000	0.895	0.700
c	—	—	—	0.075	—	—	—	—	—
d	0.075	—	—	0.135	—	—	—	0.105	0.300
PGI-1									
a	—	—	0.087	0.058	—	0.273	0.250	—	—
b	1.000	1.000	0.850	0.942	0.725	0.727	0.750	1.000	1.000
c	—	—	—	—	—	—	—	—	—
d	—	—	0.063	—	0.275	—	—	—	—
SOD-1									
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADH-1									
a	—	—	—	—	—	—	—	—	—
b	0.200	—	—	0.019	—	—	0.389	0.158	0.800
c	0.800	—	1.000	0.981	1.000	1.000	0.611	0.842	0.200

Appendix 3, continued

GDH-1

a	___	___	___	0.308	___	___	___	___	___
b	0.200	___	___	0.115	0.275	___	0.500	___	___
c	0.800	0.650	1.000	0.404	0.425	1.000	0.500	1.000	1.000
d	___	0.350	___	0.077	0.250	___	___	___	___
e	___	___	___	0.096	0.050	___	___	___	___
f	___	___	___	___	___	___	___	___	___

GOT-1

a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

GOT-2

a	___	___	___	___	___	___	___	___	___
b	0.100	0.300	0.538	0.154	0.700	0.500	0.350	0.026	___
c	0.175	0.100	___	0.038	0.050	0.068	___	___	___
d	0.725	0.600	0.462	0.808	0.250	0.432	0.650	0.974	1.000

TPI-1

a	___	0.300	___	0.173	0.300	___	___	0.132	___
b	1.000	0.700	1.000	0.827	0.700	1.000	1.000	0.868	1.000

TPI-2

a	1.000	0.850	1.000	0.288	0.450	1.000	1.000	0.974	1.000
b	___	0.150	___	0.712	0.550	___	___	0.026	___

Appendix 3, continued

Locus & Allele	POPULATION		
	BB	CC	DD
PGM-1			
a	___	___	___
b	___	___	___
c	0.750	1.000	0.909
d	0.250	___	0.909
e	___	___	___
PGM-2			
a	___	___	___
b	0.250	0.212	0.310
c	0.750	0.788	0.690
d	___	___	___
IDH-2			
a	1.000	0.848	0.955
b	___	0.152	0.045
c	___	___	___
SKD-1			
a	0.250	0.485	0.205
b	0.750	0.409	0.795
c	___	0.106	___
PGD-1			
a	___	___	___
b	1.000	0.470	0.864
c	___	0.075	___
d	___	0.455	0.136
PGI-1			
a	0.250	0.030	0.114
b	0.750	0.970	0.795
c	___	___	___
d	___	___	0.091
SOD-1			
a	1.000	1.000	1.000
ADH-1			
a	___	___	___
b	___	___	___
c	1.000	1.000	1.000

Appendix 3, continued

GDH-1

a	___	0.030	___
b	___	0.348	___
c	1.000	0.456	0.952
d	___	0.136	___
e	___	0.030	0.048
f	___	___	___

GOT-1

a	1.000	1.000	1.000

GOT-2

a	___	0.015	0.090
b	0.500	___	0.455
c	___	___	0.023
d	0.500	0.985	0.432

TPI-1

a	___	0.121	0.114
b	1.000	0.879	0.886

TPI-2

a	1.000	0.470	0.864
b	___	0.530	0.136

Appendix 4. Single locus diversity statistics (Ht, Hs, Dst, Gst) for each of the three varieties (*pubescens, parviflorum,* and *makasin*) and the species. Standard errors of the means are given in parentheses. The standard error of Gst was calculated using the jacknife method described in Weir (1990). Ht = the total amount of diversity; Hs = the average amount of expected heterozygosity; Dst = the absolute amount of variation distributed among populations; Gst = the percent of variation distributed among populations relative to the total variation.

PUB	Ht	Hs	Dst	Gst
Locus				
PGM-1	0.173	0.136	0.037	0.173
PGM-2	0.382	0.314	0.068	0.177
IDH-2	0.053	0.047	0.006	0.113
SKD-1	0.431	0.366	0.065	0.150
PGD-1	0.186	0.165	0.021	0.111
PGI-1	0.245	0.208	0.037	0.151
SOD-1	0.000	0.000	0.000	0.000
ADH-1	0.307	0.212	0.095	0.309
GDH-1	0.067	0.057	0.010	0.149
GOT-1	0.000	0.000	0.000	0.000
GOT-2	0.497	0.373	0.124	0.250
TPI-1	0.125	0.114	0.011	0.087
TPI-2	0.120	0.092	0.028	0.234
Mean	0.199	0.160	0.039	0.196
S.E.	(0.045)	(0.036)	(0.011)	(0.023)

PARV				
Locus				
PGM-1	0.113	0.096	0.017	0.148
PGM-2	0.369	0.314	0.055	0.150
IDH-2	0.054	0.042	0.012	0.229
SKD-1	0.405	0.333	0.072	0.177
PGD-1	0.023	0.022	0.001	0.051
PGI-1	0.402	0.298	0.104	0.258
SOD-1	0.000	0.000	0.000	0.000
ADH-1	0.146	0.113	0.033	0.229

Appendix 4, continued

GDH-1	0.285	0.206	0.079	0.276
GOT-1	0.000	0.000	0.000	0.000
GOT-2	0.466	0.410	0.056	0.120
TPI-1	0.064	0.046	0.018	0.276
TPI-2	0.033	0.029	0.004	0.136
Mean	0.182	0.147	0.035	0.192
S.E.	(0.049)	(0.041)	(0.010)	(0.026)

MAK
Locus

PGM-1	0.013	0.013	0.000	0.013
PGM-2	0.260	0.253	0.007	0.026
IDH-2	0.263	0.258	0.005	0.020
SKD-1	0.511	0.464	0.047	0.092
PGD-1	0.391	0.313	0.078	0.200
PGI-1	0.218	0.189	0.029	0.135
SOD-1	0.000	0.000	0.000	0.000
ADH-1	0.013	0.013	0.000	0.013
GDH-1	0.716	0.681	0.035	0.049
GOT-1	0.000	0.000	0.000	0.000
GOT-2	0.455	0.266	0.189	0.415
TPI-1	0.318	0.307	0.011	0.036
TPI-2	0.481	0.468	0.013	0.027
Mean	0.280	0.248	0.032	0.114
S.E.	(0.064)	(0.058)	(0.015)	(0.048)

SPECIES
Locus

PGM-1	0.137	0.109	0.028	0.203
PGM-2	0.370	0.310	0.060	0.163
IDH-2	0.081	0.070	0.011	0.132
SKD-1	0.452	0.370	0.082	0.181
PGD-1	0.159	0.132	0.028	0.173

Appendix 4, continued

PGI-1	0.306	0.240	0.067	0.217
SOD-1	0.000	0.000	0.000	0.000
ADH-1	0.229	0.156	0.073	0.320
GDH-1	0.229	0.162	0.067	0.292
GOT-1	0.000	0.000	0.000	0.000
GOT-2	0.492	0.380	0.113	0.229
TPI-1	0.126	0.109	0.017	0.135
TPI-2	0.182	0.107	0.074	0.409
Mean	0.213	0.165	0.048	0.224
S.E.	(0.043)	(0.035)	(0.010)	(0.021)

VITA

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Born in Nassawadox, Virginia on May 16, 1972. Graduated as Valedictorian from Nandua High School in June, 1990. Entered The College of William and Mary in August, 1990, and graduated in May, 1994 with a B.S. concentration in Biology and a minor in Psychology. August, 1994, entered the graduate program in Biology at William and Mary. In the Fall, 1997 will be attending Ohio State University to concentrate on a Ph.D. in plant systematics and conservation.