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THE POPULATION GENETIC STRUCTURE OF THE SHOWY LADY'S-
SLIPPER ORCHID (*CYPRIPEDIUM REGINAE* WALTER) IN ITS
GLACIATED AND UNGLACIATED RANGES

A Thesis

by

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Submitted to the Graduate School

Appalachian State University

In partial fulfillment of the requirements for the degree of

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Major Department: Biology

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ABSTRACT

THE POPULATION GENETIC STRUCTURE OF THE SHOWY LADY'S- SLIPPER ORCHID (*CYPRIPEDIUM REGINAE* WALTER) IN ITS GLACIATED AND UNGLACIATED RANGES

(August 2003)

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Cypripedium reginae is a terrestrial orchid with a boreal-temperate main range and a glacial-disjunct southern range primarily in the southern Appalachian Mountains. During the summer of 2001, tissue for allozyme and RAPD analysis was collected from four populations of *C. reginae* in its glaciated range of Ohio, and from five populations in its unglaciaded range. Allozyme analysis of 10 putative loci revealed a single polymorphic PGM locus in seven of nine populations. Excess heterozygosity at this locus was observed in the same seven populations. Two of thirty-one RAPD primers exhibited repeatable variation at five loci. RAPD data indicated slightly higher estimations of genetic diversity than allozymes at the species and population level, although much of this variation was distributed among populations. Allozyme data indicated low species and population-level diversity when compared to average values for

plants, other orchids and other *Cypripedium* species. Allozyme data also indicated a level of among-population differentiation similar to average values for plants and orchids. Among-population differentiation was high when compared to other *Cypripedium* species. Species and population-level allozyme diversity estimates were low when compared to estimates from plants with life-history characters similar to *C. reginae*. Among-population differentiation was not different from other plants with similar life-history characters. Higher levels of allozyme diversity were identified in populations at glaciated sites compared to populations at unglaciaded sites. Low levels of genetic variation in this species may be attributed to severe range contraction during the Wisconsin glaciation and subsequent reductions of genetic diversity due to genetic drift. Genetic drift may have been particularly intense in populations at unglaciaded sites due to infrequent appropriate habitat, isolation, and small population size. Relatively high levels of allozyme diversity in populations at glaciaded sites may be explained by the existence of *C. reginae* populations close to the glacial margin of the Laurentide ice sheet during the Wisconsin glaciation. These populations may have served as genetic reservoirs that initially colonized wetland habitats after glacial recession. RAPD data followed patterns of habitat quality. Higher levels of RAPD variation were observed in populations at sites with higher quality habitat that supported relatively large populations.

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DEDICATION

This thesis is dedicated to my wife Kellie Jacqueline Kennedy, whose beauty and grace provides me the inspiration and strength necessary to live with love, happiness, and the pursuit of dreams.

TABLE OF CONTENTS

	<u>Page</u>
List of Tables.....	x
List of Figures.....	xii
Introduction.....	1
Materials and Methods.....	19
Results.....	29
Discussion.....	57
Literature Cited.....	84
Appendix A.....	92
Appendix B.....	98
Appendix C.....	100
Vita.....	102

LIST OF TABLES

	<u>Page</u>
1. Comparison of genetic diversity statistics based on allozyme research for plants regardless of taxa, Orchidaceae, <i>Cypripedium</i> , and seven species of <i>Cypripedium</i>	13
2. The name and location of each <i>Cypripedium reginae</i> population from which collections were made in the present study. The number of stems collected from within each population is represented by n.....	20
3. Summary of genetic diversity statistics for nine populations of <i>Cypripedium reginae</i> based on allozyme analysis. The average estimate for each parameter at the population and species level is provided.....	30
4. Summary of Wright's (1978) F-statistics for a polymorphic PGM locus.....	31
5. Nei's (1978) unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) for pairwise comparisons between populations based on allozyme data.....	33
6. Average values of genetic diversity statistics based on allozyme analysis for five unglaciated and four glaciated populations of <i>Cypripedium reginae</i>	37
7. Summary of genetic diversity statistics for nine populations of <i>Cypripedium reginae</i> based on 5 polymorphic RAPD loci. The average estimate for each parameter at the population and species-level is provided.....	47
8. Nei's (1978) unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) for pairwise comparisons between populations based on RAPD data.....	50
9. Average values of genetic diversity statistics based on RAPD analysis for five unglaciated and four glaciated populations of <i>Cypripedium reginae</i>	52

10. The presence or absense of multilocus RAPD phenotypes (MLP) within nine populations of <i>Cypripedium reginae</i>	56
11. Comparison of genetic diversity statistics based on allozyme research for plants regardless of taxa, Orchidaceae, <i>Cypripedium</i> , and seven species of <i>Cypripedium</i> . Results for <i>C. reginae</i> determined in the present study are in bold.....	58
12. Comparison of average species-level and population-level genetic diversity statistics for nine populations of <i>Cypripedium reginae</i> as estimated by allozyme and RAPD analysis.....	74

LIST OF FIGURES

	<u>Page</u>
1. Distribution of forest types in eastern North America during glacial maximum of the Wisconsin glaciation 18,000 years before the present (Delcourt and Delcourt 1981).....	3
2. Distribution of forest types in eastern North America during the Wisconsin glaciation 10,000 years before the present (Delcourt and Delcourt 1981).....	5
3. Distribution of <i>Cypripedium reginae</i> according to Leur (1975).....	10
4. <i>Cypripedium reginae</i> collection site locations with corresponding sample sizes.....	22
5. Allozyme derived UPGMA dendrogram based on Nei's (1978) unbiased genetic distances.....	35
6. Distribution of PGM allele frequencies in nine populations of <i>Cypripedium reginae</i>	40
7. Observed heterozygosity and expected heterozygosity (Nei 1973) within nine populations of <i>Cypripedium reginae</i> based on allozyme analysis.....	42
8. Photograph of an agarose gel containing RAPD fragments produced from PCR using primer 28 from the UBC RAPD primer set # 1. All 43 <i>Cypripedium reginae</i> individuals included in the data set and size markers Are present in the gel. The blue arrows indicate loci that exhibited variation.....	46
9. RAPD derived UPGMA dendrogram based on Nei's (1978) unbiased genetic distances.....	49
10. Gene diversity (Nei 1973) within nine populations of <i>Cypripedium reginae</i> based on RAPD analysis.....	54

INTRODUCTION

Many plant species associated with boreal forests in their main ranges have disjunct populations south of the most southerly extent of previous glacial advances. The southerly distribution of these glacial relict plants has been described as a consequence of glacial advance and retreat, corresponding to plant species' range expansion, contraction and subsequent disjunctions (Delcourt and Delcourt 1981; Watts 1983; Pielou 1991). The most recent glacial event in eastern North America was the Wisconsin continental glaciation, at maximum between 20,000 and 18,000 ybp (Delcourt and Delcourt 1981, Mickelson et al. 1983). During this event two dramatic plant migrations likely occurred in eastern North America, one in response to the southerly advance and one in response to the northerly retreat of the Laurentide ice sheet (Figures 1, 2). During the northerly retreat many plant species remained in the southern Appalachian Mountains as ice-age relicts, maintaining disjunct, southern ranges. Therefore, it may be that disjunct southern populations are relatively ancient, have experienced fewer glaciation events than northern populations, and may have accumulated genetic variability due to isolation or experienced divergence through genetic drift.

Researchers have investigated the genetic consequences of Pleistocene climate change in populations of many North American glacial-disjunct taxa

Figure 1. Distribution of forest types in eastern North America during glacial maximum of the Wisconsin glaciation 18,000 years before the present (Delcourt and Delcourt 1981).

Figure 2. Distribution of forest types in eastern North America during the Wisconsin glaciation 10,000 years before the present (Delcourt and Delcourt 1981).

(Highton and Webster 1976; Sage and Wolf 1986; Walker 1987; Li and Adams 1989; Dolan 1994; Hawley and DeHayes 1994; Qui and Parks 1994; Wells and Bohm 1994; Lewis and Crawford 1995; Allen et al. 1996; Merila et al. 1996; Tamishiro 1996; Broyles 1998; Wallace and Case 2000; Anders and Murrell 2001). The majority of this research indicates a higher level of genetic variation in southern disjunct populations than in northern main-range populations.

Many northern boreal forest bog species have disjunct populations south of the maximum extent of the Laurentide ice sheet. Often these species are found existing together in various combinations in southern wetland habitats and on calcareous rock outcrops and cliff faces (Clebsch and Walker 1988). Some of these support plant associations that are similar to many boreal bog associations. These sites are often characterized as having a constant supply of water, similar parent material, soil close to neutral pH, a relatively open canopy, and a relatively unstable substrate leading to a frequency of disturbance that maintains the habitat's successional status while reducing encroachment of competing species. Southern Appalachian wet calcareous rock outcrops and cliff faces supporting boreal forest bog disjunct species are generally characterized by a north-facing aspect, which may help to ameliorate the temperature differences found between these low-elevation habitats at southern latitudes and those of the boreal forest.

The genus *Cypripedium* L., the lady's-slipper orchids, is composed of thirty to forty species depending on the recognition of its varieties (Luer 1975).

Cypripedium spp. are terrestrial, rhizomatous, long-lived perennial herbaceous plants often forming large clumps of vegetatively propagated stems. Kull (1998) suggests that *C. calceolus* L. clones can exist for over 100 years. *Cypripedium* leaves are plicate and pubescent on or at the base of the main axis. Flower number ranges from solitary to 12 on an inflorescence (Luer 1975).

Cypripedium flowers are highly specialized, non-rewarding and insect pollinated. Their morphology discourages self-pollination, making species of this genus obligate outcrossers (Stoutamire 1967). *Cypripedium* fruit is a dry dehiscent capsule containing thousands of tiny wind-dispersed seeds that germinate only after infection by, and subsequent symbiotic relationship with, a soil endomycorrhizal fungus (Arditti 1992).

According to Case et al. (1998), 11 species of *Cypripedium* exist in the United States and Canada, and are found in every state or province except for Florida, Hawaii, and Nevada. Most states list their *Cypripedium* species as being in some way vulnerable to extirpation. *Cypripedium reginae* Walter, the showy lady's-slipper, is listed as threatened or endangered in every southern state in which it still exists; West Virginia, Virginia, Tennessee, Arkansas. It is thought to have been extirpated from Kentucky, North Carolina, Georgia, and Alabama (Natural Heritage databases). In addition to southern populations, *C. reginae* is in decline and listed in many of its main range states.

Cypripedium reginae is a boreal-temperate forest bog species that has a main range throughout south-central and southeastern Canada, with disjunct

populations southward through Missouri and Arkansas, and extending into the southern Appalachian Mountains (Figure 3). *Cypripedium reginae* is found in wetland habitats throughout its main range and southern disjunct range. Other types of habitats in its southern disjunct range include a low-elevation, wet limestone cliff-face, and a very steep low-elevation wet limestone outcrop in northeastern Tennessee. *Cypripedium reginae* is a mid-successional community member, usually in wet, disturbed, and open sites, likely persisting over long periods of time or until successional processes act to remove it. Many *Cypripedium* species thrive in disturbed areas and are very sensitive to successional processes (Cribb 1997; Bentley 2000). Also, Cribb (1997) suggests that *C. reginae* is the most moisture-loving *Cypripedium* species in North America. *Cypripedium reginae* grows in soils of neutral to slightly basic pH, although the species is often found growing on sphagnum moss in acid wetlands, when its roots locate neutral soils below (Cribb 1997).

Cypripedium reginae is the largest native terrestrial orchid in the United States and Canada; its leaves are strongly ribbed, plicate and pubescent along the stem with one, two or rarely three flowers on the peduncle (Luer 1975). *Cypripedium reginae* does not appear to have a specific insect pollinator. However, Vogt (1990) reports *Syrphus torvus*, a syrphid fly, as this species primary pollinator and *Trichiotinus assimilis*, a flower beetle, as a minor pollinator. Stoutamire (1967) reports a medium-sized black bee successfully pollinating *C. reginae*.

Figure 3. Distribution of *Cypripedium reginae* according to Leur (1975).

Many researchers have looked at genetic variation in the Orchidaceae using a wide range of molecular tools including allozymes (Scacchi et al. 1990; Peakall and Beattie 1991; Scacchi et al. 1991; Rossi et al., 1992; Arduino et al. 1995; Sun 1996; Hollingsworth and Dickson 1997; Sun 1997; Arft and Ranker 1998; Alexandersson and Agren 2000; Ehlers and Pedersen 2000; Sharma et al. 2000; Wallace and Case 2000; Borba et al. 2001; Sun and Wong 2001; Squirrell et al. 2002; Wallace 2002), Random Amplified Polymorphic DNA (RAPD) (Sun and Wong 2001; Wallace 2002), Inter Simple Sequence Repeat (ISSR) (Smith et al. 2002), Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) (Szalanski et al. 2001) and various sequences (Gustafsson 2000; Szalanski et al. 2001). Allozyme analysis has been by far the most common molecular tool used at the plant population level. Far less research with RAPD analysis has been done, although some investigators have used RAPD analysis in concert with allozyme analysis (Sun and Wong 2001; Wallace 2002). Levels of genetic variation within populations of orchid species have been shown to vary considerably across this very diverse family. Hamrick and Godt (1996) compiled allozyme data sets from sixteen orchid species, finding the average percent polymorphic loci (P_s) to be 44.8%, the average genetic diversity within species (H_t) to be 0.137 and the average proportion of total genetic diversity at polymorphic loci among populations to be 0.087. The Orchidaceae had similar levels and distribution of genetic variation relative to other families with wind-dispersed seeds with high

P_s , high H_t and low levels of total genetic diversity distributed among populations (G_{st}). Case (2002) compiled allozyme data from 32 orchid species, finding similar levels of variation in orchid taxa as Hamrick and Godt's (1996) survey except for G_{st} levels (Table 1). Case (2002) reports a much higher average G_{st} value of 0.163 (Table 1). However, this G_{st} value is significantly less than the average G_{st} value of 0.224 reported by Hamrick and Godt (1996) for plants, regardless of taxa (Case 2002).

Cypripedium has received significant attention compared to other orchid genera. This may be attributed to their charismatic flowers, circum-temperate distribution in the northern hemisphere, and rarity. Attention to the population genetics of this genus has been no exception (Case 1993; Bornbusch et al. 1994; Case 1994; Kull 1997; Case et al. 1998; Aagaard et al. 1999; Wallace and Case 2000; Case 2002). In general, researchers have found relatively low levels of allozyme diversity in *Cypripedium* species compared to average values for orchid species (Case 2002) and other plants regardless of their taxonomic group (Hamrick and Godt 1989) (Table 1). Case (1994) surveyed allozyme variation of *C. acaule* Aiton, *C. arietinum* Brown in Aiton, *C. calceolus*, *C. candidum* Muhlenberg ex Willdenow, and *C. reginae* populations, in the Great Lakes region of the United States. *Cypripedium calceolus* contained high levels of allozyme variation at the species level, while the others contained moderate to very low levels of variation (Table 1). In fact, no variation was detected in populations of *C. arietinum* in this research or in a separate survey done by

Table 1. Comparison of genetic diversity statistics based on allozyme research for plants regardless of taxa, Orchidaceae, *Cypripedium*, and seven species of *Cypripedium*.

Taxa/Species	P _s	P _p	A _s	A _p	H _t	H _s	G _{st} /F _{st}
Plants ^a	50.5	34.2	1.96	1.53	0.149	0.113	0.224
Orchidaceae ^b	46.2	33.7	1.83	1.46	0.119	0.119	0.163
<i>Cypripedium</i>	33.3	23.9	1.38	1.28	0.071	0.058	0.150
<i>C. reginae</i> ^c	18.2	15.2	1.27	1.15	0.037	0.024	0.349
<i>C. acaule</i> ^c	46.2	34.7	1.77	1.44	0.095	0.080	0.164
<i>C. arietinum</i> ^c	0.0	0.0	1.00	1.00	0.000	0.000	0.000
<i>C. candidum</i> ^c	66.7	38.3	2.00	1.43	0.054	0.050	0.069
<i>C. parviflorum</i> ^c	75.0	62.8	2.50	1.72	0.244	0.197	0.194
<i>C. fasciculatum</i> ^d	25.0	19.5	-	1.20	0.040	0.030	0.040
<i>C. kentuckiense</i> ^e	25.0	12.5	1.33	1.15	0.050	0.042	0.182

P_s = Percent Polymorphic loci at species level.

P_p = Percent Polymorphic loci at population level.

A_s = Alleles per locus at species level.

A_p = Alleles per locus at population level.

H_t = Average species level expected heterozygosity or species level genetic diversity (Nei 1973).

H_s = Average population level expected heterozygosity or population level genetic diversity (Nei 1973).

G_{st}/F_{st} = The proportion of total genetic diversity distributed among populations (Nei 1973; Wright 1978).

^a Hamrick and Godt 1989; ^c Case 1994; ^e Case et al. 1998; ^d Aagaard et al. 1999; ^b Case 2002

Bornbusch et al. (1994). Populations of *C. reginae* contained very low species level variation, however *C. reginae* had more of its variation distributed among populations (G_{st}) than the other four species. This G_{st} value is high relative to average values for *Cypripedium* (Case 2002), other orchids (Hamrick and Godt 1996; Case 2002) and plants in general (Hamrick and Godt 1989). The yellow lady's-slipper complex, including about 18 species depending on the taxonomic recognition of varieties, has received significant attention due to taxonomic confusion within and between Eurasian and North American members (see Cribb 1997 for a detailed discussion). In addition to Case's (1994) study, relatively high levels of genetic variation have been found in eastern European *C. calceolus* (Kull 1997) and in North American varieties of *C. parviflorum* (Wallace and Case 2000). However, lower levels of variation were found in populations of *C. kentuckiense*, a rare member of the North American yellow lady's-slipper complex (Case et al. 1998). In addition, Aagaard et al. (1999) found very low levels of allozyme variation in *C. fasciculatum* Kellog ex S. Watson and low levels of genetic divergence between populations.

In contrast to the findings by most researchers of higher levels of genetic variation in southern disjunct populations versus northern main-range populations, Wallace and Case (2000) found that northern populations of the large North American yellow lady's-slipper, *C. parviflorum* var. *pubescens* contained higher levels of allozyme variation than their southern counterparts. They also found that the southern small yellow lady's-slipper, *C. parviflorum*

var. *parviflorum* contained less allozyme variation than the northern yellow lady's-slipper *C. parviflorum* var. *makasin*.

Hamrick and Godt (1989) reviewed 653 plant allozyme studies to determine how allozyme diversity is partitioned in plant species and to what extent eight key life-history characters (taxonomic status, life form, geographic range, regional distribution, breeding system, seed dispersal, mode of reproduction, successional status) account for levels and distribution of genetic variation. They found that on average, 50% of plant species' loci are polymorphic and 34% of loci are polymorphic within an average population. Average species level diversity was 15% ($H_t = 0.15$) and average population diversity was 11%. Seventy-eight percent of allozyme variation at polymorphic loci occurred within populations. Within species, these eight characters accounted for 24% of the variation in genetic diversity. Geographic range and life form explained 32% and 25 % of this variation respectively, while breeding system and seed dispersal each explained 17%. Within populations, these characters accounted for 28% of the total variation in genetic diversity. Breeding system explained 33% of this variation, geographic range 28%, life form 13%, taxonomic status 12%, and dispersal mechanism accounted for 11% (Hamrick and Godt 1989). A similar but less thorough review by Nybom and Bartish (2000) determined how a similar set of life history characters help to explain the level and distribution of RAPD variation found by other researchers in plant taxa. Nybom and Bartish (2000) found across many studies that RAPD

analysis provides higher estimations of population genetic diversity as estimated by Nei's (1973) gene diversity (H), and higher levels of among population differentiation as estimated by Nei's (1978) G_{st} than estimated with similar statistics using allozymes (Hamrick and Godt 1989). However, Nybom and Bartish (2000) and Hamrick and Godt (1989) found very similar trends in the correlation of life history characters and genetic parameters. Hamrick and Godt (1989) found higher correlations among these life history characters, likely due to the much smaller sample size of RAPD studies (Nybom and Bartish 2000). RAPD data indicated that life form, geographic range, breeding system and successional status all significantly correlate with population differentiation. However, only breeding system and successional status accounted for a significant level of within population variation in genetic diversity. One obvious difference between these two studies is the correlation between geographic range and genetic diversity. Hamrick and Godt (1989) found that allozymes indicate that widespread taxa have significantly higher within-population diversity (H_e) than either narrowly distributed taxa or endemic taxa and that endemic taxa have significantly lower levels of within-population diversity (H_e) than widespread or narrowly distributed taxa. RAPD data suggested no correlation between within-population diversity and geographic range (Nybom and Bartish 2000). However, RAPDs indicate that widespread and regional taxa had significantly more between-population diversity (G_{st}) than endemic taxa. This was not significant in Hamrick and Godt's (1989) study; in fact they

found the lowest levels of population differentiation in widespread taxa and the highest in endemic taxa.

The objective of this research is to expand the understanding of post-glacial plant population dynamics by examining the level and distribution of genetic variation in a boreal forest glacial relict plant species, *C. reginae*, in its glaciated and unglaciated range. To address the research questions and hypotheses, allozyme and RAPD analysis were conducted on individuals sampled from populations of *C. reginae* from its northerly main range and across its southern Appalachian Mountain disjunct range. The first research question addressed was whether or not levels of genetic variability were greater in populations at unglaciated sites compared to populations at glaciated sites. Second, what amount of allozyme and RAPD variation exists at the species level and how is it distributed within and among populations? Third, do any other geographic patterns exist in the allozyme or RAPD data in this species? Finally, do the levels and distribution of allozyme and RAPD variation in *C. reginae* fall into the range that others have found for *Cypripedium* species, and do they agree with findings of Hamrick and Godt (1989, 1996), and Nybom and Bartish (2000) when compared to other plants with similar key life history characters? The hypotheses addressed in this research were:

1. N₀: No allozyme or RAPD variation will be detected in populations of *Cypripedium reginae*.

N_a: Allozyme or RAPD variation will be detected in populations of *Cypripedium reginae*.

- If allozyme or RAPD variation is detected in populations of *C. reginae*, then higher levels of variation will be found in southern disjunct populations due to loss of variation in northern populations from founder effect events during northern recolonization.

2. N₀: Populations are maintained by asexual reproduction with clustered genotypes.

- Asexual reproduction may lock in genetic variation through avoidance of inbreeding in small isolated populations (Walker, 1987).

N_a: Populations are not maintained by asexual reproduction with clustered genotypes.

- Populations are maintained by sexual reproduction with a random distribution of genotypes representing sexual recombination.

MATERIALS AND METHODS

Tissue collection

During the summer and fall of 2000, leaf material was collected from nine *Cypripedium reginae* populations. Five of these populations were at unglaciated sites, two from Tennessee, one from Virginia, and two from West Virginia. Four were from glaciated sites in Ohio (Table 2; Figure 4). Plant stems collected were selected to represent the distribution of purported individuals across each population. More than one stem was sampled from multiple clumps per population to test for the origin of clumps via sexual or asexual reproduction. Samples were acquired by removing a single leaf from the main axis of the stem. The leaf was placed into a labeled plastic bag and deposited in a cooler containing ice. Upon returning to the laboratory, the samples were divided in half; one-half was immediately ground for allozyme analysis, the other half was placed into a – 80° C freezer until DNA was extracted.

Allozyme data collection

Electrophoresis of proteins from all 227 individuals was conducted in 12% starch gels. Ten allozyme loci representing nine enzymes were resolved

Table 2. The name and location of each *Cypripedium reginae* population from which collections were made in the present study. The number of stems collected from within each population is represented by n.

Population	Location	n
Powell River (PR)	Claiborne Co., TN	42
Watauga River (WR)	Carter Co., TN	8
Dismal Creek (DC)	Giles Co., VA	3
Big Draft (BD)	Greenbrier Co., WV	33
Abe Run (AR)	Tucker Co., WV	21
Cedar Bog Memorial (CBM)	Champaign Co., OH	38
Gott Fen (GF)	Portage Co., OH	30
Beck Fen (BF)	Portage Co., OH	24
Holden Arboretum (HA)	Geauga Co., OH	28
Total =		227
Average =		25.2

Figure 4. *Cypripedium reginae* collection site locations with corresponding sample sizes.

on three gel/electrode buffer systems, similar to Case (1994). Allozyme loci AAT-1 and AAT-2 (EC 2.6.1.1), GDH (EC 1.4.1.2), ME (EC 1.1.1.40), PGI (EC 5.3.1.9), and TPI (EC 5.3.1.1) were resolved on a lithium-borate pH 8.3 gel/electrode buffer system (Crawford 1982); IDH (EC 1.1.1.42), SKD (EC 1.1.1.25), and 6-PGDH (EC 1.1.1.4.4) were resolved on a histidine-citrate pH 7.0 gel/electrode buffer system (Gottlieb 1981); PGM (EC 2.7.5.1) was resolved on tris-citrate/sodium-borate pH 8.0/8.6 gel/electrode buffer system (Poulik 1957). Staining procedures for GDH, IDH, MDH, PGI, PGM, TPI, SKD, and 6-PGDH followed the agarose overlay procedures of Soltis et al. (1983); AAT followed the standard staining procedures described by Soltis et al. (1983); ME followed the staining procedures described by Werth (1985). Digital images of gel slices were taken using a Nikon Coolpix 5000 camera. Gel slices stained using the agarose overlay procedure were fixed according to Soltis et al. (1983). Gel slices stained using standard staining recipes were fixed according to Werth (1985).

Allozyme data analysis

Population genetic diversity estimates from allozyme data were calculated with the computer shareware program POPGENE (Yeh et al. 1997). Percent polymorphic loci (P), alleles per locus (A), and observed (H_o) and expected heterozygosity or overall estimation of genetic diversity (H) (Nei 1973) were calculated at the species and population level. Wright's (1978) fixation

index (F_{is}) was calculated for each population as a measure of heterozygote deficiency or excess when compared to expectations of a randomly mating population.

Wright's (1978) F-statistics were calculated, using the approach of Weir (1990), over the entire sample. F-statistics are used to measure the deviation from random mating by estimating the deficiency of heterozygotes that are expected in a randomly mating population relative to subpopulations (F_{is}), and the total sample (F_{it}) (Nei 1987). F_{st} is an estimation of the level of genetic differentiation among subpopulations (Nei 1987).

To address relationships between populations, Nei's (1978) unbiased measure of genetic distance and identity were calculated for each between population pairwise-combination and used to produce a UPGMA dendrogram.

RAPD data collection

DNA was isolated using the CTAB total DNA isolation procedure of Doyle and Doyle (1987). Individuals from five populations were selected to screen DNA template-RAPD primer combinations for variation, four from Beck Fen (BF), and three from Cedar Bog Memorial (CBM), Big Draft (BD), Powell River (PR), and Watauga River (WR). The resulting isolations were screened against 31, 10-base pair oligonucleotide primers (UBC primer set #1, The University of British Columbia, Nucleic Acid-Protein Service Unit, primer numbers 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22,

23, 24, 25, 27, 28, 29, 30, 31, 58, and 65). Total volume for each reaction was 25 μ l. Polymerase Chain Reactions (PCR) were run in Applied Biosystems' Gene Amp PCR system 9700. PCR parameters were according to Bowditch et al. (1993), with an initial 1-minute denaturation at 94° C followed by 45 cycles of denaturation at 94° C for 1 minute, annealing at 36° C for 1 minute, extension at 72° C for 2 minutes, and a single final 7-minute extension at 72° C. Each screening reaction contained 1 Amersham puReTaq™ Ready-to-go™ PCR bead, 5 μ l Primer (15 mM), 12.5 μ l DNA solution (1/100 DNA isolation to sterile H₂O), and 5.5 μ l sterile H₂O. DNA from five individuals from each population and three from the Dismal Creek population (DC) were isolated, and concentrations quantified using a fluorometer. These individuals were selected to represent the geographic range of samples within each population and were used in the data analysis. Reagents used during data collection reactions were from Sigma's CORE-T core kit. Each data reaction using primer 11 contained 0.4 μ l *taq* polymerase (2 units), 2.5 μ l 10x PCR buffer with MgCl₂ and 1.5 μ l MgCl₂ solution (3.0 μ M MgCl₂), 0.75 μ l dNTP solution (300 μ M each), 2.55 μ l PCR grade water, 3.30 μ l primer 11 (9.9 mM), and 14 μ l dilute DNA template solution (22.5 ng). Primer 28 data reactions contained 0.4 μ l *taq* polymerase (2 units), 2.5 μ l 10x PCR buffer with MgCl₂ (1.5 μ M MgCl₂), 0.5 μ l dNTPs (200 μ M each), 4.3 μ l PCR grade water, 3.3 μ l primer 28 (9.9 mM), and 14 μ l dilute DNA template solution (22.5 ng).

Fragments were separated in a 1.5% agarose TBE gel according to Anders and Murrell (2001). Electrophoresis was conducted at 40 mA using an Owl Scientific Inc. OSP-250L power supply in an Owl Separation Systems Inc. D3 gel box with a 13 cm long mold fitted with a 50 well comb. The entire 43-sample data set was run on a single gel that included a negative control lane and four size marker lanes spaced as evenly as possible across the gel. Sample and negative control wells contained 6 μ l of PCR product, and 2 μ l of loading buffer composed of bromphenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v), and sucrose (40% w/v). Electrophoresis was stopped when the bromphenol blue marker reached the end of the gel mold. The gel was stained in ethidium bromide/TBE solution (5ug/ml) for 30 minutes and destained for 10 minutes (Anders and Murrell 2001). Fragments were visualized on the UV 400-M UV Transluminator (International Biotechnologies, Inc., New Haven, CT) and analyzed using the Alpha Innotech Digital Imaging and Analysis System (Alpha Innotech Corp., San Leandro, CA).

RAPD data analysis

To ensure reliability and confidence in the fragments produced from the PCR, only primers demonstrating reproducibility and variability were scored by hand and used in the data analysis. Fuzzy or unclear bands were excluded from the data analysis. Bands produced from the same primer/template combination among individuals and of the same molecular weight were

considered to be from the same locus. Calculations of genetic diversity statistics based on RAPD data among published studies are not consistent (Nybom and Bartish 2000). Often calculations include only loci that are polymorphic within at least one population across an entire sample (Nybom and Bartish 2000, Wallace 2002). In others, all loci resulting from a primer that demonstrates the presence of at least one polymorphic locus are included (Nybom and Bartish 2000; Sun and Wong 2001). In order to most accurately make comparisons between the allozyme and RAPD analyses, the later approach was used to calculate estimations of genetic diversity in the present study. This approach makes it possible to most accurately compare allozyme and RAPD data in the present study since monomorphic loci are included in the analysis of allozyme data. At the species level and within each population, percent polymorphic loci (P), alleles per locus (A), and estimations of gene diversity using Nei's (1973) gene diversity (h) and Shannon's Information Index (I) (Lewontin 1972) were calculated using POPGENE (Yeh et al. 1997).

RAPD markers are analyzed as dominant markers so that allele frequencies are determined by the presence or absence of a specific band. As a consequence of being unable to determine heterozygosity with a dominant marker, traditional F-statistics, calculated based on allelic and genotypic frequencies, were not employed. However, population differentiation can be determined using other methods, which unlike F-statistics, are based on allele frequencies only. To estimate the average level of genetic variation distributed

among populations, G_{st} (Nei 1973) was calculated using POPGENE (Yeh et al. 1997). Similar measures of the distribution of genetic variation were calculated using Shannon's Information Index (I) (Lewontin 1972), where the proportion of genetic variation within populations is equal to the average population diversity (I_p) divided by the species level diversity (I_s), and the proportion of genetic variation partitioned among populations is $(I_s - I_p)/I_s$ (Wolff and Morgan-Richards 1999).

Nei's (1978) unbiased measure of genetic distance and identity were calculated between each population and used to produce a UPGMA dendrogram by POPGENE (Yeh et al. 1997).

RESULTS

Allozyme analysis

Species-level variation

Nine of the eighteen enzyme systems surveyed in the allozyme analysis were not used in the data collection of this study due to lack of activity or poor resolution of the enzyme. Ten loci were scored within the remaining nine enzymes. Among the ten allozyme loci surveyed, nine were monomorphic across all individuals collected from all nine populations. One PGM locus was polymorphic in seven of the nine populations sampled, with a total of three purported alleles for this locus. For the entire sample, 10% of the loci were polymorphic (P_s) and an average of 1.20 alleles per locus (A_s) was found (Table 3). Expected heterozygosity is often used as an overall measure of species and population-level genetic diversity. Average observed (H_o) and expected heterozygosity (H_i) for the total sample, across all loci were 0.076 and 0.051 respectively (Table 3). H_o and H_i for the PGM locus alone for the entire sample were 0.757 and 0.515 respectively.

Among-population variation

Wright's (1978) F-statistics calculated for the polymorphic PGM locus on this study's sample (Table 4) indicates that 21.2% ($F_{st} = 0.212$) of the total

Table 3. Summary of genetic diversity statistics for nine populations of *Cypripedium reginae* based on allozyme analysis. The average estimate for each parameter at the population and species level is provided.

Population	N	P	A	H_o	H	F_{is}
Powell River (PR)	42	10.0	1.10	0.038	0.031	-0.235
Watauga River (WR)	8	0.0	1.00	0.000	0.000	NA
Dismal Creek (DC)	3	0.0	1.00	0.000	0.000	NA
Big Draft (BD)	33	10.0	1.10	0.097	0.050	-0.941
Abe Run (AR)	21	10.0	1.10	0.100	0.050	-1.000
Cedar Bog Memorial (CBM)	38	10.0	1.10	0.076	0.050	-0.536
Gott Fen (GF)	30	10.0	1.20	0.090	0.054	-0.682
Beck Fen (BF)	24	10.0	1.20	0.088	0.051	-0.700
Holden Arboretum (HA)	28	10.0	1.20	0.093	0.059	-0.576
Average	25.2	7.8	1.11	0.065	0.038	-0.667
Species	227	10.0	1.20	0.076	0.051	-0.472

P = Percent Polymorphic loci.

A = Alleles per locus.

H_o = Observed heterozygosity.

H = Expected heterozygosity or estimation of genetic diversity (Nei 1973).

F_{is} = Deviation from random mating relative to subpopulations and an estimation of heterozygosity excess or deficiency Wright (1978).

Table 4. Summary of Wright's (1978) F-statistics for a polymorphic PGM locus.

Locus	F_{is}	F_{it}	F_{st}
PGM	-0.689	-0.332	0.212

F_{is} = The deviation from random mating relative to subpopulations.

F_{it} = The deviation from random mating relative to relative to the total sample.

F_{st} = The level of genetic differentiation among subpopulations.

sample's genetic diversity is distributed among populations. A high level of excess heterozygosity at the PGM locus is indicated by the highly negative F_{is} value of -0.689 (Table 4).

Nei's (1978) unbiased measure of genetic distance varied among pairwise population comparisons, ranging from 0.000 between the WR and DC populations, to 0.684 between the WR and HA populations and between the DC and HA populations (Table 5). The UPGMA dendrogram (Figure 5) produced from Nei's (1978) pairwise genetic distance estimates (Table 5) indicates two main clusters. One cluster includes the PR, WR, and DC populations. The other cluster includes the remaining populations, BD, AR, CBM, GF, BF, and HA.

Within population variation

The average population in this study contained 7.8% observed polymorphic loci (P_p) and an average of 1.11 alleles per locus (Table 3). Since WR and DC were fixed at all loci, they contained 0.0% polymorphic loci and an average of 1.00 allele per locus. PR, BD, AR, and CBM contained two alleles at the PGM locus, resulting in 10.0% observed polymorphic loci and an average of 1.1 alleles per locus. The GF, BF, and HA populations contained three alleles at the PGM locus, resulting in 10.0% polymorphic loci and an average of 1.2 alleles per locus (Table 3). At the population level, the average expected heterozygosity (H_s) across all loci was 0.038 (Table 3) and 0.383 for the PGM

Table 5. Nei's (1978) unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) for pairwise comparisons between populations based on allozyme data.

Populaiton	PR	WR	DC	BD	AR	CBM	GF	BF	HA
Powell River (PR)	-	0.975	0.972	0.870	0.857	0.893	0.760	0.907	0.685
Watauga River (WR)	0.026	-	1.000	0.731	0.711	0.763	0.589	0.782	0.505
Dismal Creek (DC)	0.026	0.000	-	0.731	0.711	0.763	0.589	0.782	0.505
Big Draft (BD)	0.139	0.313	0.313	-	1.010	1.006	0.989	1.006	0.948
Abe Run (AR)	0.155	0.341	0.341	-0.009	-	1.006	0.996	1.006	0.958
Cedar Bog Memorial (CBM)	0.113	0.270	0.270	-0.006	-0.006	-	0.978	1.008	0.933
Gott Fen (GF)	0.274	0.529	0.529	0.012	0.004	0.023	-	0.975	0.996
Beck Fen (BF)	0.098	0.246	0.246	-0.006	-0.006	-0.008	0.025	-	0.933
Holden Arboretum (HA)	0.378	0.684	0.684	0.054	0.043	0.069	0.004	0.069	-

Figure 5. Allozyme derived UPGMA dendrogram based on Nei's (1978) unbiased genetic distances.

locus alone. In every polymorphic population, observed levels of heterozygosity (H_o) were higher than expected (H_e). These H_e values ranged from 0.059 in the HA population to 0.031 in the PR population (Table 3). This is also indicated by Wright's (1978) fixation index (F_{is}), where every polymorphic population has a negative F_{is} value (Table 3). F_{is} ranged from -1.00 in the AR population, where all samples were heterozygous at PGM, to -0.235 in the PR population where 38.1 % of the samples were heterozygous at PGM (Table 3). It is important to note that every sample from the two West Virginia populations, BD and AR, were heterozygous at PGM, except for one sample in the BD population which was homozygous for the slowest PGM allele, Pgm-3. Therefore, every West Virginia sample except for one had a single multilocus allozyme genotype.

Macro-geographic variation

All of the population genetic parameters estimated here using allozyme analysis were found to indicate higher average levels of genetic variation in populations at glaciated versus unglaciated sites (Table 6). The average value for percent polymorphic loci was higher in populations at glaciated sites ($P = 10.00$) than in populations at unglaciated sites ($P = 6.00$) (Table 6). The average value for alleles per locus was higher in populations at glaciated sites ($A = 1.18$) than in populations at unglaciated sites ($A = 1.06$) (Table 6). The average value for average observed heterozygosity was nearly twice as high in populations at glaciated ($H_o = 0.087$) versus populations at unglaciated sites (H_o

Table 6. Average values of genetic diversity statistics based on allozyme analysis for five unglaciated and four glaciated populations of *Cypripedium reginae*.

	P	A	H _o	H
Unglaciated	6.0	1.06	0.047	0.026
Glaciated	10.0	1.18	0.087	0.054
Average	7.8	1.11	0.065	0.038
Species	10.0	1.20	0.076	0.051

P = Percent Polymorphic loci.
A = Alleles per locus.
H_o = Observed heterozygosity.
H = Expected heterozygosity or estimation of genetic diversity (Nei 1973).

= 0.047) (Table 6). The average value for expected heterozygosity across all loci was twice as high in populations at glaciated sites (H = 0.054) than in populations at unglaciated sites (H = 0.026) (Table 6).

Geographic patterns were also observed for PGM allele frequencies and levels of expected heterozygosity (H_e) among populations. Pgm-3 generally decreased in frequency in populations from south to north, while the frequency of the fastest-migrating allele, Pgm-1, generally increased in populations from south to north (Figure 6). The intermediate-migrating allele, Pgm-2, was present only in the three most northern populations in Ohio (i.e. GF, BF, and HA) and occurred at relatively low frequencies (Figure 6). The samples collected from two of the most southern populations in Tennessee and Virginia (WR and DC) were fixed at Pgm-3. Expected levels of heterozygosity also generally increased among pairwise population combinations from south to north (Figure 7). In most cases the highest genetic distance (Nei 1978) are between populations at the northern and southern extremes of the sampling range. For example, the highest genetic distance estimates are between two of the three most northern populations in Ohio (i.e. GF and HA) and two of the three most southern populations in Tennessee (i.e. WR and DC), HA x WR, HA x DC, GF x WR, GF x DC (Table 5). Relatively high levels of genetic distance are also found between the PR and the three most northern populations (i.e. GF, BF, HA) and between BF and the three most southern populations (i.e. PR, WR, DC). The UPGMA dendrogram (Figure 5) separates the populations at

Figure 6. Distribution of PGM allele frequencies in nine populations of *Cypripedium reginae*.

Figure 7. Observed heterozygosity and expected heterozygosity (Nei 1973) within nine population of *Cypripedium reginae* based on allozyme analysis.

glaciated sites from the populations at unglaciated sites, except for the two West Virginia populations that cluster with populations at glaciated sites.

Micro-geographic variation

Three of nine populations (i.e. WR, DC, AR) contained a single allozyme genotype. In most cases, where multiple samples were taken from a single suspected genet (1 clump of stems), a single PGM genotype was found. However, 25.8% of these suspected genets contained more than one PGM genotype. Other than a general pattern of observing the same genotypes within a single suspected genet, no large-scale clustering of genotypes within populations was observed since most individuals were at the same heterozygous genotype at a single PGM locus.

RAPD analysis

Species-level variation

Of the 31 RAPD primers screened, only two produced repeatable variation among the 16 individuals screened. Most of these 31 primers produced clear monomorphic banding patterns; the others resulted in very limited or poorly resolved banding activity, likely due to poor template/primer/reactant interactions. The two primers that produced repeatable variation were primers 11 and 28 from the UBC RAPD primer set.

Across the entire data set of 43 individuals, primer 11 produced 13 bands, three of which were variable, and primer 28 produced 10 bands, two of which were variable (Figure 8). These five variable characters produced a total of thirteen multilocus RAPD phenotypes (Table 7). The percent polymorphic loci for the entire sample (P_s) was 21.7%. Alleles per locus (A) at the species level were 1.22 (Table 7). Nei's (1973) gene diversity (h_t) and Shannon's Information index (I_s) estimated species level genetic diversity at 0.061 and 0.093 respectively (Table 7).

Among population variation

Nei's (1973) G_{st} was 0.384, indicating that 38.4% of the genetic variation for the entire sample was distributed among populations as indicated by RAPD analysis. Shannon's Information Index I (Lewontin 1972) estimated this proportion to be 42.0%.

The UPGMA dendrogram indicates two main clusters (Figure 9) based on Nei's (1978) unbiased genetic distance calculations (Table 8); the top cluster contains the PR, AR, BF, CBM, and GF populations, the bottom cluster contains the WR, DC, BD, and HA populations.

Within population variation

The average population contained 8.2% polymorphic loci, 1.10 alleles per polymorphic locus, and 3.2 multilocus RAPD phenotypes (MLP) (Table 7).

Figure 8. Photograph of an agarose gel containing RAPD fragments produced from PCR using primer 28 from the UBC RAPD primer set # 1. All 43 *Cypripedium reginae* individuals included in the data set and size markers are present on the gel. The blue arrows indicate loci that exhibited variation.

Table 7. Summary of genetic diversity statistics for nine populations of *Cypripedium reginae* based on 5 polymorphic RAPD loci. The average estimate for each parameter at the population and species-level is provided.

Population	N	P	A	h	I	MLP
Powell River (PR)	5	13.0	1.13	0.051	0.074	3.0
Watauga River (WR)	5	8.7	1.09	0.030	0.045	3.0
Dismal Creek (DC)	3	0.0	1.00	0.000	0.000	1.0
Big Draft (BD)	5	4.4	1.04	0.020	0.029	2.0
Abe Run (AR)	5	8.7	1.09	0.028	0.043	3.0
Cedar Bog Memorial (CBM)	5	21.7	1.22	0.080	0.118	5.0
Gott Fen (GF)	5	13.0	1.13	0.063	0.088	4.0
Beck Fen (BF)	5	8.7	1.09	0.030	0.045	4.0
Holden Arboretum (HA)	5	8.7	1.09	0.030	0.046	4.0
Average	4.8	8.2	1.10	0.037	0.054	3.2
Species	43	21.7	1.22	0.061	0.093	13.0

P = Percent Polymorphic loci.

A = Alleles per locus.

h = Nei's (1973) gene diversity.

I = Shannon's Information index (Lewontin 1972).

MLP = Number of multilocus phenotypes.

Figure 9. RAPD derived UPGMA dendrogram based on Nei's (1978) unbiased genetic distances.

Table 8. Nei's (1978) unbiased measures of of genetic identity (above diagonal) and genetic distance (below diagonal) for pairwise comparisons between populations based on RAPD data.

Populaiton	PR	WR	DC	BD	AR	CBM	GF	BF	HA
Powell River (PR)	-	0.824	0.907	0.967	0.969	0.950	0.943	0.972	0.947
Watauga River (WR)	0.194	-	0.943	0.911	0.617	0.958	0.849	0.685	0.931
Dismal Creek (DC)	0.098	0.059	-	0.978	0.706	0.889	0.793	0.749	0.990
Big Draft (BD)	0.034	0.093	0.022	-	0.829	0.912	0.847	0.875	0.999
Abe Run (AR)	0.031	0.483	0.349	0.187	-	0.845	0.902	1.004	0.779
Cedar Bog Memorial (CBM)	0.051	0.043	0.118	0.093	0.169	-	1.031	0.880	0.900
Gott Fen (GF)	0.059	0.164	0.232	0.166	0.103	-0.031	-	0.923	0.809
Beck Fen (BF)	0.028	0.378	0.290	0.134	-0.004	0.128	0.080	-	0.815
Holden Arboretum (HA)	0.054	0.072	0.010	0.001	0.250	0.106	0.212	0.205	-

The DC population was monomorphic at all RAPD loci, with all three samples having the same multilocus RAPD phenotype. The CBM population contained 21.7% polymorphic loci, 1.22 alleles per locus, and 5 multilocus phenotypes (Table 7). The PR and GF populations both contained 13.0% polymorphic loci and 1.13 alleles per locus. While GF contained 4 multilocus phenotypes, PR contained only 3 (Table 7). The WR, AR, BF and HA populations all contained 8.70% polymorphic loci and 1.09 alleles per locus. The HA population contained 4 multilocus phenotypes where the WR, AR, and BF populations each contained only 3 (Table 7). The BD population contained 4.3% polymorphic loci, 1.04 alleles per locus, and 2 multilocus phenotypes (Table 7). The average population-level genetic diversity estimates, h_s (Nei 1973) and I_p (Lewontin 1972) were 0.037 and 0.054 respectively (Table 7). The values of h and I ranged from 0.080 and 0.118 respectively in the CBM population to 0.00 for both estimates in the DC population (Table 7).

Macro-geographic variation

All of the population genetic parameters estimated here using RAPD analysis were found to indicate higher average levels of genetic variation in populations at glaciated versus unglaciated sites (Table 9). However, these parameters did not provide a clear pattern of increasing genetic diversity from south to north (Figure 10). Rather, habitats supporting the largest populations and having an open canopy contain the highest levels of genetic variation (i.e.

Table 9. Average values of genetic diversity statistics based on RAPD analysis for five unglaciated and four glaciated populations of *Cypripedium reginae*.

	P	A	h	I	MLP
Unglaciated	7.0	1.07	0.026	0.038	2.40
Glaciated	13.1	1.13	0.051	0.074	4.25
Average	8.2	1.10	0.037	0.054	3.2
Species	21.7	1.22	0.061	0.093	13.0

P = Percent polymorphic loci.
A = Alleles per locus.
h = Estimation of genetic diversity (Nei 1973).
I = Shannon's Information Index (Lewontin 1972).
MLP = Number of multilocus phenotypes.

Figure 10. Gene diversity (Nei 1973) within nine populations of *Cypripedium reginae* based on RAPD analysis.

DISCUSSION

Allozyme analysis

Species-level variation

Hamrick and Godt (1989) reviewed allozyme data from 653 studies and address the relationship that life history characters play in shaping the genetic structure of plant populations. Hamrick and Godt (1989) found that among the eight life-history characters included, geographic range accounts for the greatest proportion of species-level genetic diversity. At the population level, plant breeding system and geographic range accounts for the greatest proportion of genetic diversity, while breeding system helps to explain the highest proportion of variation among populations.

The allozyme data from this study indicates low genetic diversity at the species level. This is indicated by relatively low estimates of percent polymorphic loci, alleles per locus, and expected heterozygosity, when compared with Hamrick and Godt's (1989) average values from other plants regardless of taxa, average values for orchid species (Case 2002), and averages for other *Cypripedium* species (Table 11). In addition, all the estimates of genetic variation found in this study are much lower than Hamrick and Godt's (1989) average estimates of genetic variation for plants with life-history characteristics (i.e. monocots, long-lived herbaceous perennials,

Table 11. Comparison of genetic diversity statistics based on allozyme research for plants regardless of taxa, Ochidaceae, Cypripedium, and seven species of *Cypripedium* . Results for *C. reginae* determined in the present study are in bold.

Taxa/Species	P _s	P _p	A _s	A _p	H _t	H _s	G _{st} /F _{st}
Plants ^a	50.5	34.2	1.96	1.53	0.149	0.113	0.224
Orchidaceae ^b	46.2	33.7	1.83	1.46	0.119	0.119	0.163
Cypripedium	33.3	23.9	1.38	1.28	0.071	0.058	0.150
C. reginae	10.0	7.8	1.20	1.11	0.051	0.038	0.212
C. reginae ^c	18.2	15.2	1.27	1.15	0.037	0.024	0.349
C. acaule ^c	46.2	34.7	1.77	1.44	0.095	0.080	0.164
C. arietinum ^c	0.0	0.0	1.00	1.00	0.000	0.000	0.000
C. candidum ^c	66.7	38.3	2.00	1.43	0.054	0.050	0.069
C. parviflorum ^c	75.0	62.8	2.50	1.72	0.244	0.197	0.194
C. fasciculatum ^d	25.0	19.5	-	1.20	0.040	0.030	0.040
C. kentuckiense ^e	25.0	12.5	1.33	1.15	0.050	0.042	0.182

P_s = Percent Polymorphic loci at species-level.
P_p = Percent Polymorphic loci at population-level.
A_s = Alleles per locus at species-level.
A_p = Alleles per locus at population-level.
H_t = Average species-level expected heterozygosity or species-level genetic diversity (Nei 1973).
H_s = Average population-level expected heterozygosity or population-level genetic diversity (Nei 1973).
G_{st}/F_{st} = The proportion of total genetic diversity distributed among populations (Nei 1973; Wright 1978).
^a Hamrick and Godt 1989; ^c Case 1994; ^e Case et al. 1998; ^d Aagaard et al. 1999; ^b Case 2002

regional distribution, animal outcrossed, wind-dispersed seeds, sexual and asexual reproduction, mid-successional) similar to *C. reginae*.

Low levels of genetic diversity are also reported for other *Cypripedium* species (Table 11). An extreme example is found in Bornbusch et al. (1994), where no allozyme variation was detected at 27 putative loci across an average of 36 *C. arietinum* individuals per locus from a Massachusetts population. Similarly, Case (1994) investigated allozyme diversity in *C. arietinum* and found a complete lack of variation across 11 putative allozyme loci at four Michigan populations. Case (1994) observed similar overall levels of allozyme variation in three Michigan *C. reginae* populations as the present study. Case (1994) identified two variable putative allozyme loci, one PGM locus and one AAT locus, where the present study identifies one variable putative PGM locus and two monomorphic putative AAT loci, resulting in higher percent polymorphic loci observed in Case's (1994) study (Table 11). Alleles per locus (1.27) are slightly higher in Case's (1994) study than the 1.20 alleles per locus in the present research. However, expected heterozygosity (Nei 1973) at the species level is higher in the present study ($H_t = 0.051$) than in Case's (1994) ($H_t = 0.037$). The higher estimate of expected heterozygosity in the present study may be reflective of this study's larger sample that covers a larger extent of the geographic range of *C. reginae*. The present study indicates that *C. reginae* has species level allozyme variation that falls within the range of other

estimates within the genus (Bornbusch et al. 1994; Case 1994; Case et al. 1998; Aagaard et al. 1999) (Table 11).

Case (1994) attributes the lack of species-level genetic variation in *C. reginae* to glacial disturbances that are responsible for genetic bottlenecks in ancestral populations caused by range contractions and the infrequent availability of appropriate southern habitat. Case (1994) observed that *Cypripedium* species that presently maintain extensive southern geographic ranges and have relatively nonspecific ecological requirements, maintain higher levels of genetic variation than *Cypripedium* species with restricted southern geographic ranges and relatively specific ecological needs. This pattern may indicate that intense genetic drift was experienced by *Cypripedium* species with relatively specific ecological needs is due to a lack of appropriate southern habitat during glacial advance (Case 1994). *Cypripedium arietinum*, for example, may follow this pattern. Its entire geographic distribution is currently in previously glaciated areas near to the Wisconsin glacial maximum and two allozyme surveys (Bornbusch et al. 1994; Case 1994) have not detected variation in this species.

Low levels of genetic variation in this species may be attributed to glacial disturbance and subsequent reductions of genetic diversity due to bottlenecks and genetic drift. This research supports the idea that populations in the unglaciated range may have experienced a reduction in genetic diversity due to genetic drift, which may have been a product of severe range

contraction, small population sizes, infrequent appropriate southern habitats and isolation. However, the allozyme data from this study does not support the hypothesis that populations at northern sites have experienced further reductions in genetic diversity as a consequence of glacial retreat and northward migration of *C. reginae*. A similar pattern is reported by Wallace and Case (2000), where higher levels of allozyme variation are observed in populations of *C. parviflorum* var. *pubescens* at glaciated versus unglaciated sites. Also, Wallace and Case (2000) observed higher levels of variation in populations of the northern *C. parviflorum* var. *makasin* than that seen in populations of the southern *C. parviflorum* var. *parviflorum*. Wallace and Case (2000) explain this pattern by suggesting a northern refugium for *C. parviflorum* varieties during glaciation. They further suggest that populations at unglaciated southern sites experienced a reduction in genetic diversity due to genetic drift caused by small population sizes and reproductive isolation. Case (1994; 2002) suggests that prime habitat in previously glaciated areas may have been created for *Cypripedium* species after the northward retreat of glacial ice. This may have been especially true for *C. reginae*. Glacial recession may have left behind open wetland areas that are similar to many habitats where this species is found today in Ohio and across its range. The allozyme data indicates low levels of genetic variation in populations at previously unglaciated, southerly sites, and an increase in diversity in populations at previously glaciated,

northerly sites. During the Wisconsin Glaciation, this species may have existed close to the margin of the Laurentide Ice Sheet, and at least as far south as Tennessee and Arkansas. The two most southern extant *C. reginae* populations remain in northern Arkansas. According to a recent phylogenetic study (Cox 1995) and morphological study (Cribb, 1997), the two species most closely related to *C. reginae* are *C. flavum*, a vicarious species from eastern China (Sing-Chi 1983), and *C. passerinum*, found through much of Canada and Alaska (Cribb 1997). The habitats of these three species have many similarities. Cribb (1997) describes the habitat of *C. flavum* as open woods and scrub, and margins of glacial torrents, with damp, shallow soils, and abundant leaf litter. *Cypripedium passerinum* is found as far north as the Arctic Circle in moist, rich, coniferous forests; in deep ravines of tundra; on the margins of lakes and streams; in talus slopes, and gravel outwashes; usually on neutral or acidic soils in sphagnum moss, coniferous humus, or limestone gravel (Cribb 1997). These observations suggest that these three species' habitats are ecologically similar and that *C. flavum* and *C. passerinum* thrive close to or on glacial margins and tundra. Also, in the present study, the higher levels of allozyme variation in populations at glaciated sites may reflect their natural history of occupying habitats close to the glacial margin during the Wisconsin Glaciation. Those populations close to the glacial margin may have experienced fewer reductions from genetic drift than their more southerly ranging counterparts. Therefore, these northern populations may be more

representative of more abundant and diverse ancestral populations at the glacial margin that were positioned to recolonize a previously glaciated landscape with the recession of the Wisconsin ice sheets.

Among population variation

Although low levels of allozyme diversity were found in the present study, the diversity observed is largely found partitioned among populations as indicated by relatively high levels of F_{st} (Table 11). The F_{st} value found here (0.212) is higher than those reported for other species within the genus and is lower only than that found by Case (1994) for *C. reginae* (0.349) (Table 11). This F_{st} value is also high when compared to the average value for orchids (Case 2002) (Table 11), and is only slightly lower than the average value for plants, regardless of taxa (Hamrick and Godt 1989) (Table 11). The F_{st} value observed in this study is not different from Hamrick and Godt's (1989) average estimates of population genetic differentiation (G_{st}) for plants with similar life history characters (i.e. monocots, long-lived herbaceous perennials, regional distribution, animal outcrossed, wind-dispersed seeds, sexual and asexual reproduction, mid-successional). These results indicate that *C. reginae* distributes its genetic variation among populations in a pattern similar to that observed in other plant species with comparable life histories and to a greater degree than other *Cypripedium* species with similar life-history characters.

An interesting observation from this data indicates that populations at glaciated sites are genetically similar to each other as compared to southern populations which have diverged from each other to a greater extent. The average genetic distance (Nei 1978) between populations at unglaciated sites ($D = 0.165$) is more than five times greater than the average genetic distance (Nei 1978) between populations at glaciated sites ($D = 0.030$). In the southern extent of its range, the life history of *C. reginae* may have included long periods of population isolation in terms of reproduction and recruitment, due to the limited availability of suitable habitats and increased competition from other species better adapted to warmer conditions. Appropriate habitat is more continuous in the glaciated range of *C. reginae*'s distribution, which may facilitate gene flow from recruitment of wind-dispersed seeds from nearby populations. Also, northern populations may be experiencing gene flow through pollen dispersal among proximate populations. However, disjunct southern populations are much older than populations at glaciated sites. Therefore, the effects of genetic drift on disjunct southern populations may be much greater and may help to explain the greater differences among southern disjunct populations when compared to populations at glaciated sites. The level of population differentiation found in populations at glaciated sites from the present study is in contrast to Case's (1994) results. The relatively high level of population differentiation found in Case (1994) may indicate that dramatic founder effect was experienced in newly colonized sites after glacial recession.

Relatively diverse populations close to the margin of the Laurentide Ice Sheet may have initially colonized previously glaciated sites with only a fraction of its genetic information represented in a new population. Some sites may have been colonized with individuals having rare or unique polymorphisms. Differences among populations may have been exaggerated over time by the proliferation of vegetatively reproduced stems within populations.

Within population variation

Average population-level genetic diversity estimates for *C. reginae* in this study are low and are comparable to species-level diversity estimates. Following a similar pattern as species-level diversity, average population-level diversity is low when compared with Hamrick and Godt's (1989) average values from other plants regardless of taxa, average values for orchid species (Case 2002), and average values for other *Cypripedium* species (Table 11). Also, similar to species-level diversity patterns, all of the average population-level estimates of genetic variation found in this study are much lower than Hamrick and Godt's (1989) average estimates of genetic variation for plants with life-history characteristics similar to *C. reginae* (i.e. monocots, long-lived herbaceous perennials, regional distribution, animal outcrossed, wind-dispersed seeds, sexual and asexual reproduction, mid-successional). This is consistent with Hamrick and Godt's (1989) report that allozyme estimates of population-level genetic diversity accurately reflect species-level genetic variation.

Average within-population estimates of genetic diversity from Case (1994) indicate twice the level of percent polymorphic loci ($P_p = 15.20$) than those observed in the present study ($P_p = 7.80$), and slightly higher alleles per locus ($A_p = 1.15$) than the present study ($A_p = 1.11$) (Table 11). Average expected heterozygosity ($H_s = 0.024$) in Case's (1994) study are lower than those of the present study ($H_s = 0.038$) (Table 11). Case (1994) reports one of three Michigan *C. reginae* populations having an excess of heterozygosity at a single variable PGM locus, as expressed by the fixation index ($F_{is} = -0.468$). The present study indicates a more extensive excess of heterozygosity at a single variable PGM locus. Excess heterozygosity was observed in seven of nine populations surveyed in this study. Exceptions include the two smallest populations, which are also two of the three most southern populations (i.e. WR and DC). High levels of observed heterozygosity in other *Cypripedium* spp. is reported by Kull (1997) who found very high average levels of heterozygosity at three AAT loci in eight populations of *C. calceolus* L. in Estonia. In the present study, the high frequency of heterozygous combinations may suggest a combination of selection for heterozygotes at this PGM locus and frequent vegetative reproduction of stems within populations. When only well separated samples are analyzed, very similar estimates of F_{is} , and all other estimates of genetic variation, are found. This indicates that the sampling strategy did not bias F_{is} , or any other estimates of genetic diversity. However, *C. reginae* is known to vegetatively reproduce from a rhizome and is known to form colonies of

over 1,000 stems (Cribb 1997). The contraction and fragmentation of a population over time may lead to the appearance of many distinct clumps that were once a continuous genet. Therefore, it is not clear how many genetically distinct individuals were sampled in this study and it is not known to what extent vegetative propagation is responsible for high levels of excess heterozygosity. Also, it appears that climate may select for frequencies of different heterozygous genotypes in northerly versus southerly populations based on predominance of certain alleles, where the frequency of Pgm-3 is higher in southerly populations, Pgm-1 is higher in northerly populations, and Pgm-2 is only present in the three most northerly populations (i.e. GF, BF, HA). On the other hand, historic geographic patterns of genetic drift may have affected the frequency of PGM alleles in *C. reginae*. Selection for heterozygotes and vegetative reproduction do explain genotypic or allelic frequencies within the two most southerly populations (i.e. WR and DC). In these populations, all of the samples are homozygous for Pgm-3. The high level of observed homozygosity in these populations is likely not a product of the small sample size from these populations, since the collection at the DC population represents a census ($n = 3$) and the collection from WR represents a near census ($n = 8$). The fixation of alleles at these two population's PGM locus may be due to genetic drift and small population size.

Macro-geographic patterns

Allozyme data indicates genetic differences between populations at unglaciated and glaciated sites. This is indicated by the observation that populations at glaciated sites (i.e. CBM, GF, BF, HA) generally have higher estimates of genetic diversity than populations at unglaciated sites (i.e. PR, WR, DC, BD, AR) (Table 3). Also, higher average population estimates of genetic diversity are found between populations at glaciated versus unglaciated sites (Table 6). Changes in PGM allele frequencies from north to south, and the resulting differences in homozygous combinations in populations at glaciated versus unglaciated sites indicates divergence between glaciated and unglaciated populations as well. Genetic separation between these two groups is further indicated by the observation that the highest level of genetic distance is found between populations at the northern and at the southern extremes of the total sampling (Table 5).

The three most southern populations of *C. reginae* sampled in this study (i.e. PR, WR, DC) have the lowest level of genetic diversity as estimated by expected heterozygosity (H_e) (Nei 1973) (Table 3). H_e generally increases from south to north in every population except in the case of two northern Ohio populations where there is a slight decrease of H_e from 0.054 to 0.051 in GF and BF respectively. Additionally, two of the three most southern populations (i.e. WR and DC) are the only populations that are fixed for a single allele per locus (Table 3). On the other hand, the four populations at glaciated sites (i.e.

CBM, GF, BF, HA) have the four highest values of Nei's (1973) overall genetic diversity, H_e (Table 3). Also, the three most northern populations (i.e. GF, BF, HA) contain an additional allele, Pgm-2, not found at unglaciated sites, raising the alleles per locus observed in these northern populations to the highest levels among all nine populations (Table 3).

Not only do allele frequencies within populations vary from south to north, also homozygous combinations differ among populations at glaciated versus unglaciated sites. Even though homozygous combinations are uncommon within most populations, there appears to be a geographic pattern for these combinations, which may be driven by selection for certain alleles at different latitudes or genetic drift. For example, over 50% of the samples from the three most southern populations are homozygous for the Pgm-3 allele. Also, even though Pgm-1 homozygotes are rare in populations at unglaciated sites, no Pgm-1 homozygotes exist in populations at unglaciated sites. Therefore, the homozygous Pgm 1-1 combination may be particularly unfit in populations at unglaciated sites while the homozygous Pgm 3-3 may be relatively unfit in populations at glaciated sites. Possibly most striking is the very high frequency of the Pgm 3-1 genotype within every population except for WR in Tennessee and DC in Virginia. The fixation of the PGM locus in WR and DC may be a product of random genetic drift and isolation, selection, or vegetative reproduction. Eanes (1999) reports that multiple researchers demonstrate selection pressure on allozyme loci of metabolic pathways. For

example, Eanes et al. (1993) found low levels of sequence divergence of a G6PD gene between populations of *Drosophila melanogaster* and *D. simulans*, while dramatically different G6PD allozyme allele frequencies between populations of these species north and south of the Saharan desert were found, indicating geographic selection for G6PD alleles.

Nei's (1978) genetic distance and identity values indicate low levels of divergence between geographically proximate populations of *C. reginae* in this study (Table 5). However, these genetic distances indicate a relatively high level of separation between populations at glaciated (i.e. CBM, GF, BF, HA) versus unglaciated sites (i.e. PR, WR, DC, BD, AR) (Table 5). The genetic distances and topology of the dendrogram are driven by the difference in frequency of the Pgm-1 and -3 alleles, as well as the presence of the Pgm-2 allele in the three most northern populations (i.e. GF, BF, HA). Since the two West Virginia populations cluster with the populations at glaciated sites, the geographic separation as indicated by the dendrogram is not strong. The West Virginia populations (i.e. BD and AR) are within this cluster because the frequencies of the Pgm-1 and Pgm-3 alleles in these populations are similar to those found in northerly populations. Within this large cluster, the BD, AR, CMB, and BF populations cluster separately from GF and HA (Figure 6). The BF population does not cluster with GF and HA, to which it is most geographically proximate, because of its very low frequency of Pgm-2 and nearly equal distribution of the Pgm-1 and Pgm-3 alleles. This equal

distribution is observed in the BF, CBM, AR, BD cluster. The DC and WR populations cluster with the PR population based on the fixation of Pgm-3, which is the most common allele in the PR population. The populations BD, AR, CBM, BF, BD, HA in the lower cluster of the dendrogram are all within the Ohio River drainage (Figure 6). Populations of the top cluster (i.e. PR, WR, DC) are within the Tennessee River drainage, except for DC, which is within the Ohio River drainage (Figure 6). This pattern may be explained by genetic drift at PGM due to isolation into separate river drainages. A similar pattern for PGM alleles in different river drainages is observed in Sloan (1994). This research investigates the population genetic structure of Ruth's golden aster, *Pityopsis ruthii* (Small) Small, a rare composite herb that is restricted to exposed phyllite and greywacke rock outcrops of the Hiwassee and Ocoee River drainages in Tennessee. Sloan (1994) finds differences at a PGM locus between populations on the Hiwassee compared to those in the Ocoee River drainages in Tennessee, indicating that these populations may be experiencing drift due to separation into different river drainages.

Micro-geographic variation

The within-population sampling strategy of collecting multiple stems from a single suspected genet in this study provides evidence that many of the clumps contain more than one genetically distinct individual. Other authors (Case 1993; Case 1994; Aagard et al. 1999; Wallace and Case 2000) indicate

that their methodology includes sampling from only one stem from a single suspected genet to avoid sampling the same individual more than once. This is a concern since many *Cypripedium* species form large clumps of vegetatively reproduced genets (Cribb 1997). The sampling strategy employed in the present research was used because without physically unearthing rhizomes in the field, it is difficult to be certain what demarcates a single genet. Also, since some of the *C. reginae* populations included in this study were small, and previous work by Case (1994) indicated low levels of genetic variation in *C. reginae*, it was important to sample as many individuals as possible. Over 25% of the clumps from which multiple stems were sampled, contained more than a single genetically distinct individual. Since some stems were identified as being genetically distinct and only a few centimeters apart, it is suggested that a sampling strategy that includes collecting from multiple stems within a single clump of *Cypripedium* species may provide more accurate levels of within and among population diversity.

RAPD analysis

Species-level variation

Although numerous investigations of *Cypripedium* species using allozyme analysis have been conducted, research aimed at addressing genetic variation of *Cypripedium* species using PCR-based techniques at the population

level is limited. Further, few researchers have investigated genetic variation in the Orchidaceae using RAPDs, and even fewer in *Cypripedium*.

The low species-level genetic diversity that was estimated by RAPD analysis is likely due to many of the same forces that drove low levels of variation in the allozyme analysis. It is well documented that RAPD analysis often indicates higher overall levels of genetic variation in plants than allozyme analysis in plants (Baruffi et al. 1995; Chan and Sun 1997; Huang et al. 1998; Wolff and Morgan-Richards 1999; Cole and Kuchenreuther 2001; Sun and Wong 2001). For example, Sun and Wong (2001) investigated both allozyme and RAPD variation in three orchid species, *Zeuxine gracilis*, *Z. strateumatica*, and *Eulophia sinensis*, and found higher levels of RAPD variation across all genetic diversity parameters in all three species. RAPDs also indicate greater genetic variation than allozymes across all parameters in Wallace's (2002) survey of the eastern prairie fringed orchid, *Platanthera leucophaea*. In fact, Sun and Wong (2001) found at least 11 times more RAPD variation than allozyme variation within the three orchid species investigated, as estimated by Nei's gene diversity (1973). Wallace (2002) found twice the amount of RAPD variation in *P. leucophaea* than allozyme variation, as estimated by Nei's gene diversity (1973).

In the present research, RAPD analysis indicated higher levels of genetic diversity than allozyme analysis (Table 12). For example, RAPDs indicated

Table 12. Comparison of average species-level and population-level genetic diversity statistics for nine populations of *Cypripedium reginae* as estimated by allozyme and RAPD analysis.

	P _s	P _p	A _s	A _p	H _t	H _s	G _{st} /F _{st}
Allozymes	10.0	7.8	1.20	1.11	0.051	0.038	0.212
RAPDs	21.7	8.2	1.22	1.10	0.061	0.037	0.384

P_s = Percent Polymorphic loci at species-level.
P_p = Percent Polymorphic loci at population-level.
A_s = Alleles per locus at species-level.
A_p = Alleles per locus at population-level.
H_t = Average species-level expected heterozygosity or species level genetic diversity (Nei 1973).
H_s = Average population level expected heterozygosity or population level genetic diversity (Nei 1973).
G_{st}/F_{st} = The proportion of total genetic diversity distributed among populations (Nei 1973; Wright 1978).

more than twice the percent polymorphic loci (P_s), slightly higher alleles per locus, and higher gene diversity (Nei 1973) (Table 12). Shannon's Information Index calculates substantially higher levels of overall genetic variation than Nei's gene diversity (1973) in the present study. This is also the case for Sun and Wong (2001), and Wallace (2002). Caution is necessary in interpreting these data, however, as Nei (1987) suggests that the biological significance of Shannon's Information Index is uncertain.

In the present study, estimates of genetic variation were derived from polymorphic loci and monomorphic RAPD loci only from primers indicating at least one polymorphic locus. In contrast to estimating variation based on polymorphic loci only, substantially lower estimates of genetic variation were estimated in the present study. This method of data analysis is similar to the allozyme data analysis, providing comparable results among these two methods. Higher levels of RAPD variation are found in the present study as compared to that found in the allozyme analysis even though the total sample size is much smaller than the allozyme sample size. The higher estimation of variation in the RAPD analysis may be due to many factors. First, the genetic code is redundant, meaning that a single nucleotide change at a specific codon may not change the amino acid produced. In addition to electrophoretically-silent nucleotide variation, electrophoretically-silent amino acid variation has also been identified in plants and animals. Eanes (1999) suggests that by sequencing allozyme loci, many authors researching animals and plants have

demonstrated that many genes have substantially more amino acid substitutions than are apparent using allozyme electrophoresis (Cooke and Oakeshott 1989; Hasson and Eanes 1996; Miyashita et al. 1996). RAPDs on the other hand, produce relatively small DNA fragments based on very short primers, producing results that are more sensitive to sequence-level changes. Therefore, many polymorphisms go undetected using traditional allozyme analysis, and RAPD data may provide a more accurate reflection of sequence divergence (Eanes 1999). Second, it is widely known that different parts of the genome change at different rates and for different reasons (Nei 1987). In contrast to evidence of selection pressure on many allozyme loci, many authors (Baruffi et al. 1995; Nybom and Bartish 2000; Cole and Kuchenreuther 2001) suggest that in general, selection does not act on RAPD loci, or does so minimally. This is likely because many RAPD fragments are produced from non-coding regions such as micro- and minisatellites. These regions are also thought to be less conserved and therefore may inherently contain higher levels of variation than coding sequences. Third, allozyme analysis is limited to mainly enzymes involved in cellular respiration, so the number of loci that can be utilized is limited (Eanes 1999, Nybom and Bartish 2000). This is in contrast to RAPDs, which have the ability to produce bands as products from a nearly unlimited number of primers with different sequences. Finally, unlike allozyme studies, most RAPD studies do not report the number of total loci screened, and like the present study, do not include all monomorphic loci from calculations of

diversity estimates (Nybom and Bartish 2000). This alone may be capable of inflating estimates of genetic diversity based on RAPD analysis compared to estimates based on allozyme analysis.

Among-population variation

Shannon's Information Index provided an estimation of population genetic subdivision that is similar to Nei's (1973) G_{st} . RAPDs are dominant markers, and therefore do not detect heterozygosity. Nei's (1973) estimation of gene diversity can be affected by RAPDs inability to detect heterozygosity. However, Shannon's index is used here since it is not affected to the degree that Nei's (1973) estimates are by the dominant nature of RAPDs (Dawson et al. 1995). Both of these estimations identify a high level of genetic variation distributed among populations in this study. Shannon's Information Index estimates that 42.0% of the total variation is distributed among populations, and is very similar to estimates by G_{st} (Nei 1973) at 38.4%. Both of these estimations are substantially higher than the F_{st} (Nei 1978) estimation from the allozyme analysis of 21.2%. It is important to note, however, that both of the techniques provide evidence that a high proportion of the total genetic variation identified in this study, across the entire sample, is distributed among populations. The increased level of among population differentiation observed in the RAPD analysis relative to the allozyme analysis may indicate that RAPD

analysis is a more sensitive tool for detecting genetic diversity among populations of *C. reginae* than allozyme analysis.

Within-population variation

Estimates of average population-level diversity by allozyme and RAPD analysis are more similar to each other than allozyme and RAPD based estimates are at the species-level (Table 12). Percent polymorphic loci was the only estimate that was higher in the RAPD analysis than in the allozyme analysis. Both alleles per locus and gene diversity (Nei 1973) were the same.

Even though only five variable characters are identified, and only five individuals from most populations are used in the RAPD analysis, multilocus phenotypes (MLP) provide evidence that RAPD analysis is a good tool for determining within-population structure. In the present study RAPDs are able to identify unique multilocus phenotypes within some populations (Table 10). This indicates that RAPDs may be used to identify genetically distinct individuals. Therefore, with a larger population sample size, specific analysis of *C. reginae* within-population genetic structure may be possible with RAPDs. For example, using allozymes, one genotype was indicated in the WR and DC populations, and two genotypes in the CBM population. However, RAPD analysis identifies at least three genotypes in the WR population, and five in the PR population (Table 10). A single multilocus RAPD phenotype is observed among the three DC samples, also indicating one genetically distinct individual.

These results have conservation implications. First, these populations are very small and may be easily lost as a result. This is significant since there are less than 10 known populations in the southern Appalachian Mountains. Since the DC population's discovery in 1993, the number of plants has been reduced from 18 to the three stems sampled in the present study (Personal communication, Stanley Bentley). The remaining stems are likely the same individual as indicated by the allozyme and RAPD results of the present study and by the observation that these stems are centimeters apart. Second, because two of the three multilocus phenotypes (MLP) identified in the WR population, and one of the five identified in the PR population are unique and found in none of the other populations in this study (Table 10). Since the WR and PR populations are found at the extreme southern portion of *C. reginae*'s distribution, it may be important to retain its unique genetic variation. This species is likely generally sensitive to warm climates. Unique genetic variation in southern Appalachian Mountain populations at low elevations may indicate that these populations are more adapted to warm climates than their northern counterparts. A tolerance of relatively warm temperatures may be an important adaptation for a species that will likely experience warmer climates in the future as a consequence of global climate change.

Nyblom and Bartish (2000) report that, in their survey of RAPD analyses, they found no association between sample-size per population or number of polymorphic loci and any genetic diversity statistics. However, few studies were

surveyed where five or fewer individuals per population were used and even fewer were surveyed that used five or fewer variable characters in their analysis. Nyblom and Bartish (2000) recommend avoiding low population sample-sizes and recommend using at least 50-100 polymorphic loci in most situations. A RAPD analysis with a larger within-population sample size than that of the present study may indicate higher species-level and population-level genetic variation in *C. reginae*. An increase in polymorphic characters in a RAPD analysis of *C. reginae* populations may increase the detection of differences among individual samples within and among populations. An increase in sample size and number of polymorphic characters may provide greater resolution of among population relatedness.

Macro-geographic patterns

The data from the RAPD analysis supports some of the same observations as the allozyme analysis does. RAPD data also indicate differences between populations at glaciated versus unglaciated sites. This is supported by the observation that average values for all of the genetic diversity estimates are higher in populations at glaciated sites versus populations at unglaciated sites (Table 9). This is also supported by the observation that most multilocus genotypes are restricted to either populations at glaciated sites or populations at unglaciated sites (Table 10). However, the dendrogram (Figure 9) indicates a general lack of close relationships between geographically

proximate populations. The RAPD data however indicate that levels of genetic variation within populations may correspond to habitat quality. Populations PR, CBM, GF, at sites that have all of the characteristics common to high quality habitat *C. reginae* sites, including an open canopy, also contain the highest level of genetic variation. Not surprisingly, these sites also support the largest populations.

A lack of geographic patterns in the RAPD data, and correspondence with patterns found in the allozyme data may be due to the small number of individuals used and the small number of available polymorphic loci in the RAPD analysis.

Summary

This study detected a very limited amount of allozyme and RAPD variation in populations of *C. reginae*. An unexpectedly small number of polymorphic RAPD loci were recognized during the screening process of this research. Relatively clear geographic patterns were revealed from the allozyme data even though they were predominantly based on a single polymorphic locus. Despite the low levels of detectable allozyme and RAPD variation, both data sets suggested differences between populations at glaciated versus unglaciated sites. Additional allozyme loci may help to determine whether certain PGM alleles and genotypes are more fit in certain geographic locations, and if historical fragmentation and contraction of genets within populations are

responsible for the excess heterozygosity observed in this study. An increase in the number of individuals used per population in the RAPD analysis also has the potential of clarifying within-population patterns that were not investigated here due to small within-population sample size. The observation that RAPDs were able to distinguish individuals of *C. reginae* using multilocus phenotypes, where as the allozyme analysis did not, indicates that RAPDs may be a better tool for determining within-population structure in this species. Therefore, an efficient method for determining within-population relationships in *C. reginae* may be to increase the number of individuals in a RAPD analysis. This may be preferable to screening for further variable allozyme loci as the probability of finding variable allozyme loci may be low. Additionally, RAPDs were able to identify unique multilocus phenotypes, corresponding to unique genotypes, in glaciated and unglaciated sites. The identification of unique genotypes in populations at unglaciated sites is particularly significant since the likelihood of these genotypes existing in other populations is unlikely due to the level of isolation and rarity of southerly populations. Unique genotypes in the southern Appalachian Mountains may be of particular conservation concern since they may have the ability to provide a tolerance to higher temperatures. This tolerance may be significant for a species that will likely experience global warming. Regardless, the protection of unique genetic material is important. Also, RAPD data indicated high levels of genetic diversity in large populations found in high-quality habitat. Therefore, the maintenance of populations,

primarily in ephemeral southerly habitats, may be an effective conservation strategy for protecting genetic diversity in *C. reginae* populations.

Allozymes indicated higher genetic diversity in populations at glaciated sites versus populations at unglaciaded sites. RAPD data indicated higher average genetic diversity among populations at glaciaded sites versus populations at unglaciaded sites. This pattern was unexpected since most of the investigations into patterns of genetic variation in glacial-relict species have indicated higher levels of variation in populations at unglaciaded sites versus those at glaciaded sites. Higher estimates of allozyme diversity in populations at glaciaded sites versus unglaciaded sites may reflect post-glacial primary succession into high-quality habitats from relatively diverse populations near the margin of the Laurentide Ice Sheet during the Wisconsin glacial recession. Populations at unglaciaded sites may have experienced intense genetic drift during glacial maximum due to the low frequency of appropriate habitat and subsequent isolation and small population size. Therefore, levels of population genetic diversity are expected to decline northward as the distance between populations and the most southern extent of the Laurentide Ice Sheet increases. Future research could effectively investigate this hypothesis by sampling populations throughout the entire range of *C. reginae*.

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Appendix A
Gel Buffer, Electrode Buffer and Enzyme Stain Protocols

Gel and Electrode Buffer Protocols

Histidine-Citrate 7.0 (Gottlieb 1981)Gel buffer:

0.02 M (4.19 g/L) histidine HCl

Titrate pH to 7.0 with 4 M NaOH

Electrode buffer:

0.4 M (117.64 g/L) citric acid tri-sodium salt

Titrate to pH 7.0 with HCl

Run gel at 75 mA for 9 hours

Lithium-Borate 8.3 (Crawford 1982)Gel buffer:

Nine parts buffer A:

0.05 M (6.06 g/L) tris-HCl

0.007 M (1.47 g/L) citric acid monohydrate

Titrate to pH 8.3 as needed

One part electrode buffer

Electrode buffer:

0.038 M (1.59 g/L) lithium hydroxide

0.188 M (11.62 g/L) boric acid

Titrate to pH 8.3 as needed

Run gel at 300 V for 4-5 hours

Tris/Citrate 8.6-Sodium/Borate 8.0 (Poulik 1957)Gel buffer:

0.076 M (9.20 g/L) Tris-HCl

Titrate to pH 8.6 with 0.005 M citric acid monohydrate

Electrode buffer:

0.300 M (18.55 g/L) Boric Acid

Titrate to pH 8.0 with NaOH

Run gel at 250 V for 5-6 hours

Enzyme Stain Protocols

All recipes that include the addition of 1% agarose are to be added as agarose overlays. Boil the 1% agarose solution in a microwave. When boiling stops, immediately add the appropriate volume to the staining solution. Quickly add the resulting solution to the gel slice and be sure to completely and evenly cover. Let the agarose overlay harden before moving it around. This helps to avoid clumping of the agarose, which may result in poor band resolution.

Aspartate aminotransferase (AAT; EC 2.6.1.1) (modified from Soltis et al. 1983)

1.0 M Tris-HCl buffer pH 8.0	5.0 ml
H ₂ O	45.0 ml
L-aspartic acid	50.0 mg
α -ketoglutaric acid	50.0 mg

Titrate pH to 8.0 with 1.0 M NaOH, then add:

Pyridoxal-5-phosphate	2.5 mg
Fast blue BB salt	50.0 mg

Stain in the dark at room temperature

Glutamate dehydrogenase (GDH; EC 1.4.1.2) (modified from Soltis et al. 1983)

1% PMS	0.1 ml
1% MTT	0.4 ml
1% NAD	0.3 ml
L-glutamic acid, monosodium salt	100.0 mg
0.1 M Tris-HCl buffer, pH 8.0	8.0 ml
1% agarose	8.0 ml

Stain in the dark at room temperature

Isocitrate dehydrogenase (IDH; EC 1.1.1.42) (modified from Soltis et al. 1983)

1% PMS	0.1 ml
1% MTT	0.4 ml
1% NADP	0.4 ml
MgCl ₂	40.0 mg
Isocitric acid, monosodium salt	10.0 mg
0.1 M Tris-HCl buffer, pH 8.0	8.0 ml
1% agarose	8.0 ml

Stain in the dark at room temperature

Malic enzyme (ME; EC 1.1.1.40) (Werth 1985)

0.2 M Tris-HCl pH 8.0	25.0 ml
1 M MgCl ₂	0.5 ml
2 M DL Malate	2.5 ml
1% NADP	0.5 ml
1% MTT	0.5 ml
1% PMS	0.2 ml

Stain in the dark at 37°C

Phosphoglucisomerase (PGI; EC 5.3.1.9) (modified from Soltis et al. 1983)

1% PMS	0.1 ml
1% MTT	0.4 ml
1% NAD	0.1 ml
0.1 M Tris-HCl buffer, pH 8.0	6.0 ml
10% MgCl ₂	1.0 ml
0.018 M fructose-6-phosphate, disodium salt	1.0 ml
Glucose-6-phosphate dehydrogenase	10.0 units
1% agarose	8.0 ml

Stain in the dark at room temperature

Phosphoglucmutase (PGM; EC 2.7.5.1) (modified from Soltis et al. 1983)

1% PMS	0.1 ml
1% MTT	0.5 ml
1% NAD	0.4 ml
0.1 M Tris-HCl buffer, pH 8.0	3.0 ml
0.05 M glucose-1-phosphate, disodium salt	2.5 ml
Glucose-6-phosphate dehydrogenase	10.0 units
0.001 M fructose-1,6-diphosphate, trisodium salt	1.0 ml
10% MgCl ₂	1.0 ml
1% agarose	8.0 ml

Stain in the dark at room temperature

6-Phosphogluconic acid (6-PGDH; EC 1.1.1.44) (modified from Soltis et al. 1983)

1% PMS	0.1 ml
1% MTT	0.4 ml
1% NADP	0.4 ml
6-phosphogluconic acid, barium salt	10.0 mg
10% MgCl ₂	0.5 ml
0.1 M Tris-HCl buffer, pH 8.5	8.0 ml
1 % agarose	8.0 ml

Add 2 ml of 0.1 M Tris-HCl buffer (pH 8.5) to the 6-phosphogluconic acid, barium salt (a total volume of 8 ml of the Tris-HCl buffer will be used), proceed with the recipe when the 6-phosphogluconic acid, barium salt is dissolved in the Tris buffer.

Stain in the dark at room temperature

Shikimate dehydrogenase (SKD; EC 1.1.1.25) (modified from Soltis et al. 1983)

1% PMS	0.1 ml
1% MTT	0.4 ml
1% NADP	0.4 ml
Shikimic acid	10.0 mg
0.1M Tris-HCl buffer, pH 8.0	8.0 ml
1% agarose	8.0 ml

Stain in the dark at room temperature

Triosephosphate isomerase (TPI; EC 5.3.1.1) (modified from Soltis et al. 1983)

1% PMS	0.1 ml
1% MTT	0.4 ml
1% NADP	1.0 ml
EDTA, tetrasodium salt, dihydrate	10.0 mg
Arsenic acid	150.0 mg
Dihydroxyacetone phosphate	1.5 mg
Glyceraldehyde-3-phosphate dehydrogenase	75.0 units
0.1 M Tris-HCl buffer, pH 8.0	8.0 ml
1% agarose	8.0 ml

Add the Dihydroxyacetone phosphate and Glyceraldehyde-3-phosphate dehydrogenase from solution in 0.1 M Tris-HCl buffer, pH 8.0. Store this solution in the refrigerator. If activity begins to fade, remix solutions.

Stain in the dark at room temperature

Appendix B
RAPD primer sequences from RAPD primer set #1, The University of British
 Columbia, Nucleic Acid-Protein Service Unit

All RAPD primer sequences are written from 5' to 3':

1. CCT GGG CTT C	17. CCT GGG CCT C
2. CCT GGG CTT G	18. GGG CCG TTT A
3. CCT GGG CTT A	19. GCC CGG TTT A
4. CCT GGG CTG G	21. ACC GGG TTT C
5. CCT GGG TTC C	22. CCC TTG GGG G
6. CCT GGG CCT A	23. CCC GCC TTC C
7. CCT GGG GGT T	24. ACA GGG GTG A
8. CCT GGC GGT A	25. ACA GGG CTC A
9. CCT GCG CTT A	27. TTT GGG GGG A
10. GGG GGG ATT A	28. CCG GCC TTA A
11. CCC CCC TTT A	29. CCG GCC TTA C
12. CCT GGG TCC A	30. CCG GCC TTA G
13. CCT GGG TGG A	31. CCG GCC TTC C
14. CCT GGG TTT C	58. TTC CCG GAG C
15. CCT GGG TTT G	65. AGG GGC GGG A
16. GGT GGC GGG A	

Appendix C
RAPD phenotype data

The presence or absence of a band at five RAPD loci that exhibited variation in the present study are presented below. The locus column indicates the primer that was used to produce each fragment, and each fragment's corresponding base-pair size. A 1 indicates band presence and a 0 indicates band absence.

Locus	Individual														
	PR			WR			DC			BD			AR		
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Primer 11 - 1298	1	1	0	1	1	0	0	0	0	0	1	0	1	1	1
Primer 11 - 582	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Primer 11 - 425	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Primer 28 - 520	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1
Primer 28 - 469	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0

VITA

Aaron H. Kennedy was born in Lancaster, Ohio, on March 8, 1976. He attended St. Bernadette Elementary School in Lancaster, Ohio, and graduated from Lancaster High School in June 1994. The following August he entered Francis Marion University. In the summer of 1999, Aaron graduated with a Bachelor of Science degree in Biology and Psychology.

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