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CONSERVATION GENETICS AND TAXONOMIC STATUS OF THE RARE KENTUCKY LADY'S SLIPPER: *CYPRIPEDIUM KENTUCKIENSE* (ORCHIDACEAE)[1]

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Cypripedium kentuckiense is a recently described rare orchid found in Arkansas (predominantly) and in eight other states. Much debate has focused on whether this taxon should be recognized as a distinct species or considered to be an extreme manifestation of the variability present in the widespread taxon *Cypripedium parviflorum* var. *pubescens*. In this study, 12 isozyme loci were analyzed for 14 populations of *C. parviflorum* var. *pubescens* and eight populations of *C. kentuckiense*. These data were used to examine the genetic similarity of these taxa, assess whether isozyme data support the continued recognition of *C. kentuckiense* as a distinct species, and assess whether a newly discovered disjunct Virginia population of *C. kentuckiense* is genetically isolated from other *C. kentuckiense* populations. The isozyme data revealed that the two taxa are very closely related with a high interspecific genetic identity. However, *C. kentuckiense* populations contain a subset of the variation present in *C. parviflorum* var. *pubescens*, and they have expected levels of heterozygosity that are one-quarter that of *C. parviflorum* var. *pubescens* populations. *Cypripedium kentuckiense* also possesses one widespread unique allele and a unique multilocus genotype. These data suggest that *C. kentuckiense* should be recognized as a distinct species, possibly of recent origin from *C. parviflorum*. Lastly, the isozyme data support the hypothesis that gene flow between the Virginia population and other populations of *C. kentuckiense* has been restricted.

Key words: conservation; *Cypripedium kentuckiense*; *Cypripedium parviflorum*; genetics; isozymes; Orchidaceae; rare species; taxonomy.

Temperate lady's slipper orchids (*Cypripedium* spp.) are among the most uncommon yet conspicuous members of the north temperate orchid flora. They are all long-lived herbaceous perennials that produce a flower with one of the three petals modified into a large pouch-like structure called the labellum. In most species, this structure aids in pollination by acting as a temporary trap for potential insect pollinators. Although the labellum is brightly colored and fragrant in some *Cypripedium* species, the presence of a nectar reward has not yet been found for any members of the genus. Once pollinated, a single flower can produce thousands of seeds, however very few seedlings are usually found within a given population. According to the most recent taxonomic interpretation of the genus in North America, 11 species are found in the United States and Canada, and each state or province contains one or more *Cypripedium* species with the exceptions of Florida, Hawaii, and Nevada. Furthermore, the vast majority of states have designated their *Cypripedium* species as endangered, threatened, special concern, or otherwise imperiled and vulnerable to extinction (Heritage databases and personal communication with Heritage botanists).

Threats to natural populations of these species are due to human encroachment on habitat, as well as centuries of exploitation by amateur collectors. As early as the beginning of the 18th century, colonists exported various species of *Cypripedium* out of the country for cultivation purposes (Correll, 1950). Currently, the collection of wild *Cypripedium* continues at levels ranging from hobbyist (personal observation at field sites) to large-scale illegal poaching and trade (Luer, 1975; Soucy, 1979; De Pauw and Remphrey, 1993; Stolzenburg, 1993). Over some large geographic areas (e.g., Great Britain) various *Cypripedium* species have even been hunted to extinction (Farrell and Fitzgerald, 1989). The attractive nature of *Cypripedium* flowers combined with technical difficulties associated with their ex situ cultivation and propagation (Luer, 1975; Case, 1987; Stoutamire, 1989), have resulted in increased collection pressure and subsequent rarity of various *Cypripedium* species. The development of seed germination techniques for cultivation has been especially difficult (Arditti, 1982; Ballard, 1987; De Pauw, Remphrey, and Palmer, 1995; Rasmussen, 1995). Therefore, most nurseries that sell *Cypripedium* species must usually replenish their stocks from wild stands (De Pauw and Remphrey, 1993).

Conservation efforts for taxa affiliated with the North American yellow lady's slippers (the *Cypripedium parviflorum* Salisb. complex) have also been encumbered by over 200 years of taxonomic debate. Most of this debate focuses on taxonomic interpretations of the vast amount of morphological, ecological, and genetic variation present in this species complex (e.g., Correll, 1950; New-

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house, 1976; Case, 1993). The debate began in 1791 when Salisbury segregated all North American yellow *Cypripedium* from the Eurasian *C. calceolus* L. and called the North American entity *C. parviflorum*. In 1802 Willdenow further segregated the North American plants into two species, *C. parviflorum* and *C. pubescens* Willd. Many additional species and subspecific segregations followed with emphasis on the delimitation of the variation present in *C. pubescens* as well as the taxonomic delimitation of *C. parviflorum* and *C. pubescens* [see Correll (1938) and Newhouse (1976) for reviews]. Most of the segregates (e.g., vars. *planipetalum* Fernald, *flavescens* DC, and *veganum* Cockerel) are not currently recognized (Sheviak, 1995). Another issue of debate has been the taxonomic rank at which these taxa are recognized (Atwood, 1985a). Correll, Fernald, and others have recognized the North American entities as varieties of one highly polymorphic species, *C. calceolus* [e.g., *C. calceolus* var. *parviflorum* (Salisb.) Fernald and *C. calceolus* var. *pubescens* (Willd.) Correll]. As currently recognized by Sheviak (1994, 1995), the North American *Cypripedium parviflorum* complex consists of three highly morphologically and genetically variable varieties of *C. parviflorum* [vars. *parviflorum, pubescens* (Willd.) Knight, and *makasin* (Farwell) Sheviak] as well as three other closely related species, *C. candidum* Muhlenb. ex Willd., *C. montanum* Douglas ex Lindley, and *C. kentuckiense* C. Reed. Although investigation and debate concerning this taxonomy (especially with regard to subspecific taxonomy of *C. parviflorum*) are still ongoing (e.g., Wallace, 1997), this paper will use the most current taxonomy of Sheviak.

Taxonomic difficulties within this group have caused rare putative taxa to be recognized previously as extreme variants of more common and widespread taxa. This problem is most evident in *Cypripedium kentuckiense* (the Kentucky or Rafinesque's lady's slipper), a recent species segregate of the *Cypripedium parviflorum* complex. Prior to its valid description (Reed, 1981), *Cypripedium kentuckiense* was considered to be either an unusual form of the common yellow lady's slipper, *Cypripedium parviflorum* var. *pubescens* (e.g., Correll, 1950) or a distinct species [e.g., Soukup (1977) named the species *C. daultonii* Soukup but did not cite a type specimen or provide a detailed description or Latin diagnosis]. The common yellow lady's slipper is the most morphologically and ecologically variable entity within the complex and it occurs in over 40 states and throughout most of Canada (Luer, 1975; Sheviak, 1995). Interpretation of its morphology is complicated by high levels of phenotypic and genotypic diversity (Case, 1993; Sheviak, 1995). Therefore, considerable confusion has also surrounded the formal recognition and circumscription of *C. kentuckiense* [see Atwood (1984) and Brown (1995) for a detailed taxonomic history of *C. kentuckiense*]. Consequently, there is still some debate as to whether this entity should receive taxonomic recognition.

Since its valid description in 1981 and subsequent publicity (e.g., Atwood, 1985b), many botanists and organizations have sponsored regular searches for additional populations of *C. kentuckiense*. Currently, 156 populations of this taxon are known with most populations (68%) occurring in only two states, Arkansas and Kentucky (Weldy et al., 1996). Other states with *C. kentuckiense* populations include Louisiana (12.2%), Tennessee (5.7%), Texas (5.1%), Oklahoma (4.5%), Alabama (2.6%), Mississippi (1.3%), and Virginia (0.6%). It is currently listed with a global rank of category 3 (very rare and local throughout its range) and a federal rank of category 2 (possibly threatened but more data on biological vulnerabilities and anthropogenic threats are needed; Department of the Interior, 1993). Most states consider it to be critically imperiled and very vulnerable to extirpation. The lack of documentation of potential threats to *C. kentuckiense* and past taxonomic confusion over morphological variation in the *C. parviflorum* complex have probably impeded the inclusion of *C. kentuckiense* in the federal endangered and threatened list.

In 1995, an exceptional range extension for *C. kentuckiense* was made when a large population of this taxon was discovered in eastern Virginia. The Virginia population is located in a habitat commonly reported for this species, a sandy stream bottom within a steep calcareous ravine. One interesting feature of this population is its size. Over 120 individuals were found in this uncharacteristically large population. In the survey of Weldy et al. (1996), population sizes of *C. kentuckiense* were typically very small (under 20 individuals). To characterize the morphology of the Virginia population, Weldy et al. (1996) conducted univariate and multivariate morphometric analyses of *C. kentuckiense* from Virginia and from western populations, and of *C. parviflorum* var. *pubescens* from several states. These analyses revealed that the Virginia population consisted of individuals that were not significantly different from more western *C. kentuckiense* individuals, but were significantly different from *C. parviflorum* var. *pubescens* individuals. Therefore, it was concluded that the Virginia population was *C. kentuckiense*. However, several individuals in this population approached the dimensions of *C. parviflorum* var. *pubescens,* and it was suggested that the Virginia population might either be introgressed with *C. parviflorum* var. *pubescens,* or be displaying signs of prolonged genetic isolation (Weldy et al., 1996).

This paper addresses the taxonomic debate concerning the status of *C. kentuckiense* as well as the level and distribution of genetic variation in *C. kentuckiense* compared to *C. parviflorum* var. *pubescens*. Specifically, isozyme variation was examined at 12 loci in eight populations of *C. kentuckiense* and 14 populations of *C. parviflorum* var. *pubescens* to address the following questions: (1) how genetically similar are *C. kentuckiense* and *C. parviflorum* var. *pubescens*? (2) do these taxa have similar levels and distributions of genetic variation? (3) do the isozyme data support the continued recognition of *C. kentuckiense* as a distinct species? and (4) does the disjunct population of *C. kentuckiense* discovered in Virginia show the effects of genetic isolation from other *C. kentuckiense* populations? The populations analyzed genetically in this study include the same set of populations used in the morphological analyses of Weldy et al. (1996). Information from both analyses should help resolve the ongoing debate over the taxonomic status of *C. kentuckiense,* as well as provide conservation biologists with basic knowledge concerning the geographic distribution of genetic variation in *C. kentuckiense*. These re-

TABLE 1. Collection location information, population sample size (*N*), percentage of polymorphic loci (P), alleles per locus (*A*), and average observed (H_{obs}) vs. average expected (H_{exp}) heterozygosity for each population of *Cypripedium parviflorum* var. *pubescens* and *C. kentuckiense* sampled.

Taxon and location	*N*	P	*A*	H_{obs}	H_{exp}
C. parviflorum var. *pubescens*					
Georgia-1; Union Co.	19	58.3	1.6	0.096	0.102
Indiana-1; Noble Co.	29	66.7	1.8	0.153	0.162
Kentucky-1; Bullitt Co.	20	41.7	1.4	0.158	0.131
Michigan-1; Chippewa Co.	22	83.3	2.1	0.183	0.231
Michigan-2; Mackinac Co.	27	83.3	2.1	0.253	0.249
Michigan-3; Emmet Co.	20	50.0	1.5	0.204	0.189
Michigan-4; Presque Isle Co.	20	83.3	2.2	0.267	0.240
Missouri-1; Wayne Co.	13	50.0	1.6	0.154	0.132
Missouri-2; Lincoln Co.	20	58.3	1.8	0.126	0.178
Missouri-3; Lincoln Co.	9	50.0	1.5	0.065	0.208
Tennessee-1; Sevier Co.	23	58.3	1.7	0.117	0.131
Virginia-2; James City Co.	19	75.0	1.9	0.184	0.211
Virginia-3; Nelson Co.	10	8.3	1.1	0.050	0.037
Virginia-4; Nelson Co.	8	41.7	1.4	0.177	0.130
Population average	18.5	57.7	1.69	0.156	0.167
Species-level value	259	83.3	2.83	0.166	0.198
C. kentuckiense[a]					
Arkansas-1	20	8.3	1.1	0.054	0.037
Arkansas-2	20	8.3	1.1	0.046	0.037
Arkansas-3	40	8.3	1.1	0.036	0.029
Arkansas-4	40	16.7	1.2	0.111	0.081
Oklahoma-1	20	16.7	1.2	0.013	0.050
Texas-1	20	16.7	1.2	0.055	0.053
Texas-2	20	8.3	1.1	0.009	0.023
Virginia-1	40	16.7	1.2	0.031	0.029
Population average	27.5	12.5	1.15	0.044	0.042
Species-level value	220	25	1.33	0.045	0.050

[a] County locations of *C. kentuckiense* are not published due to the sensitivity of this taxon to collection pressure.

sults should also aid future re-evaluations of the potential addition of *C. kentuckiense* to the federal endangered and threatened species list.

MATERIALS AND METHODS

In the spring of 1995, collections of leaf tissue for isozyme electrophoresis were made. Samples were obtained from individuals in eight populations of *C. kentuckiense* (from Arkansas, Oklahoma, Texas, and Virginia) and 14 populations of *C. parviflorum* var. *pubescens* (from Georgia, Indiana, Kentucky, Michigan, Missouri, Tennessee, and Virginia; Table 1). Sampling occurred uniformly throughout the spatial distribution of individuals in each population and over all size classes of individuals. Sample sizes per population ranged from 8 to 40 individuals, with a mean of 21.7 individuals (Table 1). When fewer than 20 individuals (i.e., genets) were found within a population, a sample was collected from every individual in the population. Individual plants within a tight clump were treated as one individual and sampled only once. For each plant sampled, ~3–6 cm^2 of leaf tissue was removed, divided among two 1.5-mL centrifuge tubes, and placed immediately on ice. Samples were subsequently packed on blue ice and shipped via an overnight carrier to The College of William & Mary, where they were frozen, unprocessed, at −75°C.

All details of the grinding, electrophoretic, and staining procedures are explained in Case (1993). Isozyme electrophoresis resolved the protein products of 12 putative loci using eight enzyme systems. Two loci were scored for phosphoglucomutase (PGM), triosephosphate isomerase (TPI), and glutamate oxaloacetate transaminase (GOT). One locus was scored for 6-phosphogluconate dehydrogenase (6PGD), isocitrate dehydrogenase (IDH), superoxide dismutase (SOD), shikimate dehydrogenase (SKD), and phosphoglucose isomerase [PGI; this enzyme is an addition to those published in Case (1993) and was resolved on the lithium borate system described in Case (1993) using the agarose overlay staining schedule in Soltis et al. (1983)]. To compare the mobilities of proteins from different populations, representative individuals from different populations were electrophoresed together on the same gel.

All statistics were calculated with the program BIOSYS-1 (Swofford and Selander, 1989) and include the percentage of polymorphic loci (P; a locus was considered polymorphic if greater than one allele was present), alleles per locus (*A*), and observed and expected heterozygosities. Each of these statistics was calculated at the species as well as population level for each taxon. Alleles per locus and heterozygosity estimates were calculated using all loci. For each polymorphic locus in each population, the exact probability of the genotypic array under the assumption of random mating was calculated. Loci were considered to show significant departure from Hardy-Weinberg equilibrium when probabilities were equal to or below 0.05. Taxonomic differentiation was evaluated using Nei's (1978) unbiased genetic identity values and Cavalli-Sforza and Edwards (1967) chord distance. The latter was chosen for use in a cluster analysis using an unweighted pair-group method with arithmetic averaging (UPGMA). The UPGMA dendrogram using Cavalli-Sforza and Edwards chord distance is reported herein because it resulted in the highest cophenetic correlation of the 13 distance and identity measures calculated by BIOSYS-1. Inbreeding coefficients and the amount of variation distributed among populations for populations within a taxon were evaluated using Wright's (1965) *F* statistics. In this analysis, F_{IS} is the estimated inbreeding coefficient due to nonrandom mating within populations, F_{ST} is the amount of variation distributed among populations, and F_{IT} is the amount of inbreeding due to the combined effects of inbreeding within populations and genetic drift among populations.

RESULTS

Of the 12 loci examined, only two (*Sod* and *Got1*) were monomorphic in all populations examined. Each remaining locus was polymorphic in at least one taxon, although *C. parviflorum* var. *pubescens* had a greater amount of genetic diversity as measured by polymorphic loci, alleles per locus, and heterozygosity at the species and population levels (Table 1). In *C. parviflorum* var. *pubescens*, the species-level percentage of polymorphic loci was 83.3% as compared to 25% in *C. kentuckiense*. Every locus that was polymorphic in *C. kentuckiense* was also polymorphic in *C. parviflorum* var. *pubescens*. *Cypripedium parviflorum* var. *pubescens* had on average 2.83 alleles per locus, whereas *C. kentuckiense* had on average only 1.33 alleles per locus. Population-level values also differed among the two taxa, and these reflected the species-level trends. For all loci except one (*Got2*), the major allele in *C. kentuckiense* was the major allele in *C. parviflorum* var. *pubescens*. Only two unique alleles were found in *C. kentuckiense*, *Pgm1-d* and *Got2-d* (Table 2). The former was found exclusively in the Virginia *C. kentuckiense* population at a population frequency of 0.075, whereas *Got2-d* was found in every *C. kentuckiense* population except the Virginia population. Furthermore, *Got2* in the Virginia population of *C. kentuckiense* contains a common *C. parviflorum* var. *pubescens* allele (*Got2-e*, at a population frequency of 0.112) that was not present in any other *C. kentuckiense* population. It should also be

TABLE 2. Allele frequencies of polymorphic loci in *Cypripedium parviflorum* var. *pubescens* and *C. kentuckiense* averaged across all populations within each species.

Locus and allele		*C. parviflorum* var. *pubescens*	*C. kentuckiense*
Pgm1	a	0.004	—
	b	0.886	0.984
	c	0.021	—
	d	—	0.016[a]
	e	0.089	—
Pgm2	a	0.266	—
	b	0.732	1.00
	c	0.002	—
Tpi1	a	0.073	—
	b	0.927	1.00
Tpi2	a	0.906	1.00
	b	0.094	—
6pgd	a	0.002	—
	b	0.892	1.00
	c	0.023	—
	d	0.083	—
Got2	a	0.017	—
	b	0.351	0.698
	c	0.010	—
	d	—	0.281[b]
	e	0.622	0.021[c]
Gdh	a	0.004	—
	b	0.962	1.00
	c	0.022	—
	d	0.012	—
Pgi1	a	0.104	0.048
	b	0.868	0.952
	c	0.002	—
	d	0.026	—
Skd	a	0.286	—
	b	0.714	1.00
Idh2	a	0.965	1.00
	b	0.033	—
	c	0.002	—

[a] Allele found exclusively in the Virginia population of *C. kentuckiense* where it is at a population frequency of 0.075.

[b] Allele found in all *C. kentuckiense* populations except the Virginia population.

[c] The Virginia population is the only *C. kentuckiense* population that contains this allele.

noted that the two alleles unique to *C. kentuckiense* remain unique even when a larger number of *C. parviflorum* populations from another study are included in the data set (e.g., $N = 30$ populations encompassing all three varieties; Wallace, 1997).

For both taxa, average observed population heterozygosities were similar to average expected population heterozygosities. However there was a fourfold mean difference among taxa in expected heterozygosities (H_{exp} for *C. parviflorum* var. *pubescens* = 0.167; H_{exp} for *C. kentuckiense* = 0.042; Table 1). Out of 12 single-locus tests for Hardy-Weinberg equilibrium in *C. kentuckiense*, there were two significant deviations involving two populations and two loci (*Got2* and *Pgi1*). *Got2* had a deficiency of observed heterozygotes in Texas-2 and *Pgi1* had an excess of observed heterozygotes in Oklahoma-1. In *C. parviflorum* var. *pubescens*, 97 total tests for deviations from Hardy-Weinberg equilibrium resulted in ten significant deviations. These deviations were spread across six loci and, with the exception of one test, were in the direction of observed heterozygote deficiency. Four populations (Missouri-2, Missouri-3, Virginia-2, and Michigan-1) had heterozygote deficiencies at two or more loci.

TABLE 3. Wright's (1978) *F* statistics for *Cypripedium parviflorum* var. *pubescens* (PUB) and *C. kentuckiense* (KEN) for each locus polymorphic in at least one species. A dash indicates the absence of an *F* value due to a monomorphic locus in the species.

	F_{IS}		F_{ST}		F_{IT}	
Locus	PUB	KEN	PUB	KEN	PUB	KEN
Pgm1	0.123	−0.081	0.197	0.066	0.296	−0.009
Pgm2	0.141	—	0.128	—	0.251	—
Tpi1	0.189	—	0.060	—	0.238	—
Tpi2	−0.110	—	0.223	—	0.138	—
6pgd	−0.094	—	0.100	—	0.016	—
Got2	0.053	−0.104	0.288	0.147	0.326	0.059
Gdh	−0.131	—	0.144	—	0.033	—
Pgi1	0.135	0.018	0.089	0.291	0.213	0.304
Skd	−0.093	—	0.122	—	0.040	—
Idh2	−0.032	—	0.108	—	0.079	—
Mean	0.032	−0.076	0.163	0.182	0.190	0.120

The overall consistency of most loci with Hardy-Weinberg expectations is reflected in the average F_{IS} values that are at or near zero (e.g., 0.032 in *C. parviflorum* var. *pubescens* and −0.076 for *C. kentuckiense*; Table 3). However, some loci in *C. parviflorum* var. *pubescens* (e.g., *Pgm2* and *Tpi1*) have higher and positive F_{IS} values (0.141 and 0.189, respectively), while other loci (e.g., *Gdh*) have lower values (−0.131). Average overall structure (F_{IT}) was slightly higher for *C. parviflorum* var. *pubescens* (0.190) than for *C. kentuckiense* (0.120). A substantial portion of the F_{IT} values in both taxa was due to allele frequency variance among populations (as measured by F_{ST}). Average F_{ST} values were 0.163 in *C. parviflorum* var. *pubescens* and 0.182 in *C. kentuckiense.*

Lastly, for comparisons among conspecific populations, Nei's genetic identity values over all loci ranged from 0.865 to 1.00 (mean value = 0.967) in *C. parviflorum* var. *pubescens* and from 0.951 to 1.00 (mean value = 0.990) in *C. kentuckiense.* Interspecific identity values were slightly lower on average (mean value = 0.939) and ranged from 0.869 to 0.982. Cavalli-Sforza and Edwards chord distances displayed similar trends. Distance values ranged from 0.097 to 0.350 (mean value = 0.196) in *C. parviflorum* var. *pubescens* and from 0.00 to 0.225 (mean value = 0.092) in *C. kentuckiense.* Interspecific distance values were higher on average (mean value = 0.279) and ranged from 0.176 to 0.353 (data for distance and identity values are not shown). The differences between intra- and interspecific values are reflected in the results of UPGMA cluster analysis. All *Cypripedium kentuckiense* populations cluster at genetic distance values of 0.149 or less, whereas *Cypripedium parviflorum* var. *pubescens* populations cluster at values of 0.247 or less (Fig. 1). The *C. kentuckiense* group joins the *C. parviflorum* var. *pubescens* group at 0.279. The cophenetic correlation for the UPGMA is 0.910, and the percentage standard deviation is 15.38.

DISCUSSION

The interspecific mean genetic identity value of 0.939 for *Cypripedium kentuckiense* and *C. parviflorum* var. *pubescens* is high compared to 0.67, which is an average

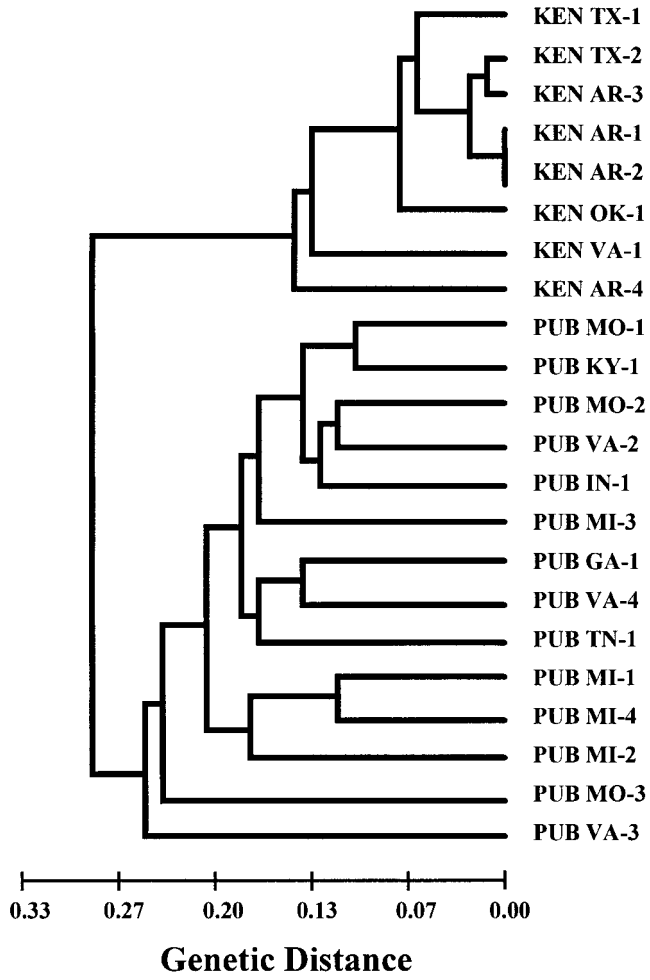

Fig. 1. UPGMA dendrogram of *C. kentuckiense* (KEN) and *C. parviflorum* var. *pubescens* (PUB) populations using Cavalli-Sforza and Edwards (1967) chord distance. Populations are indicated by the abbreviation for the state in which they were collected, followed by a population number. The cophenetic correlation is 0.910, and the percentage standard deviation is 15.38.

congeneric value calculated from a wide variety of plant isozyme studies (Gottlieb, 1977, 1981; Crawford, 1983). However, despite their high interspecific genetic identity, *C. parviflorum* var. *pubescens* and *C. kentuckiense* possess clear differences in genetic variation at the isozyme level as indicated by the distinct taxonomic groups present in the UPGMA dendrogram (Fig. 1). These genetic differences are due primarily to seven out of ten polymorphic loci which were fixed and monomorphic in *C. kentuckiense* but were polymorphic in *C. parviflorum* var. *pubescens* (Table 2). These fixed *C. kentuckiense* alleles were the highest frequency alleles found in *C. parviflorum* var. *pubescens*. Other alleles found at the three polymorphic loci in *C. kentuckiense* were either unique alleles (e.g., *Pgm1-d* and *Got2-d*) or other high-frequency alleles also present in *C. parviflorum* var. *pubescens* (e.g., *Got2-e* and *Pgi1-a*). One population of *C. parviflorum* var. *pubescens* (Virginia-3) contained relatively few polymorphic loci. Although this population had a degree of fixation commonly present in *C. kentuckiense* populations, it contained a polymorphic locus (*6Pgd*) that was never polymorphic in *C. kentuckiense* populations, and it did not contain any alleles unique to *C. kentuckiense*.

These two species differ dramatically in their observed levels of genetic variation, although there are no apparent differences in their life history characteristics that might account for these genetic differences. In a review paper, Hamrick and Godt (1989) found that a plant species' geographic range and breeding system accounted for the greatest proportion of variation in population levels of genetic diversity. Although *C. kentuckiense* and *C. parviflorum* var. *pubescens* share many biological similarities, they do differ substantially in geographic ranges and number of populations. The average population values of the percentage of polymorphic loci, alleles per locus, and expected heterozygosity for species with wide distributions (43% and 1.72 and 0.159, respectively; Hamrick and Godt, 1989) are comparable to those values found in the widespread *C. parviflorum* var. *pubescens* (57.7% and 1.69 and 0.167). In contrast, *Cypripedium kentuckiense* has population diversity values (12.5% and 1.15 and 0.042) that are closer to the mean values calculated for populations of endemic taxa (26% and 1.39 and 0.063). However, *C. kentuckiense* has a wide geographic range (from Texas to Virginia) compared to many endemic taxa included in studies of allozyme variation (e.g., Loveless and Hamrick, 1988; Purdy and Bayer, 1995).

The interspecific genetic differences among *C. kentuckiense* and *C. parviflorum* var. *pubescens* are substantially greater than the genetic differences found among varieties of *C. parviflorum*. For example, Case (1993) found no consistent allele frequency differences that distinguished varieties of *C. parviflorum* in the northeastern United States. Wallace (1997) expanded the analysis of varietal variation in *C. parviflorum* to populations in the southeastern United States and also concluded that the isozyme variation examined could not discriminate among varieties *parviflorum* and *pubescens*. The lack of diagnostic genetic differentiation among subspecific taxa is relatively common among taxa that are not allopatric or ecologically separated (Crawford and Smith, 1984; Heywood and Levin, 1984; Wolf, Soltis, and Soltis, 1991). A lack of intraspecific genetic differentiation has also been found in two other orchid species examined, *Orchis morio* (Rossi et al., 1992) and *O. papilionacea* (Arduino et al., 1995). In these studies, the authors concluded that this lack of isozyme differentiation among conspecific populations demonstrated a lack of intraspecific taxa. The isozyme results for *Cypripedium* combined with morphological differences previously observed between *C. parviflorum* var. *pubescens* and *C. kentuckiense* (Weldy et al., 1996) provide strong support for the continued recognition of *C. kentuckiense* as a distinct species.

The level of genetic divergence between *C. kentuckiense* and *C. parviflorum* var. *pubescens* bares striking similarities to another species pair in this complex that includes *C. parviflorum*. *Cypripedium candidum* is a midwestern taxon that inhabits wet prairies and fens. It is morphologically similar to *C. parviflorum*, but is smaller and has a white labellum. This taxon can be found in hybrid swarms with *C. parviflorum* in transitional habitats where the two co-occur (Actor, 1984; Klier, Leoschke, and Wendel, 1991). The *C. candidum*-*C. parviflorum* spe-

cies pair have a relatively high interspecific genetic identity (0.79) due to the occurrence in *C. candidum* of several loci that are nearly fixed for the highest frequency alleles of *C. parviflorum.* Similar to *C. kentuckiense,* there are few unique alleles in *C. candidum* (Case, 1994). For the rare taxon in both species pairs, the number of polymorphic loci, number of alleles per locus, and levels of heterozygosity are dramatically reduced and appear to be a subset of variation found in the more widespread *C. parviflorum.* For example, measures of genetic diversity at the species level in *C. kentuckiense* are approximately two- to fourfold lower than *C. parviflorum* var. *pubescens.* Similar genetic patterns for species pairs are frequently reported in the literature and are often interpreted as an indication of a progenitor-derived species association (see reviews in Crawford, 1983; Pleasants and Wendel, 1989; Edwards and Wyatt, 1994). Furthermore, the reduced level of genetic variation in the rare taxon is consistent with the occurrence of a genetic bottleneck during its evolutionary history (Leberg, 1992). However, it is usually not known whether a bottleneck occurred before or after speciation. An interesting result from the *Cypripedium* data is that the *C. parviflorum*-*C. candidum* and *C. parviflorum*-*C. kentuckiense* species pairs both possess genetic patterns consistent with progenitor-derived associations. These data are consistent with the hypothesis that *C. parviflorum* has been the progenitor of two extant taxa and that *C. kentuckiense* and *C. candidum* have a high degree of allelic similarity because they share many of the highest frequency alleles of *C. parviflorum.* These two taxa may have experienced the same genetic bottleneck prior to their phylogenetic separation or, alternatively, two independent genetic bottlenecks could have occurred. Recent DNA sequence data for a large number of *Cypripedium* taxa (including those taxa in the present study) indicate a very close affinity of *C. kentuckiense* to *C. candidum,* placing them as sister taxa in a cladistic analysis (Cox, 1994).

There has been much speculation in the literature concerning evolutionary processes in the Orchidaceae (e.g., Darwin, 1884; Garay, 1960; Dodson et al., 1969; Dressler, 1981; Benzing and Atwood, 1984; Kiester, Lande, and Schemske, 1984; Benzing, 1986; Nilsson, 1992). One of the commonly discussed themes is the likelihood that genetic bottlenecks and other forms of genetic drift contribute to the orchid speciation process (e.g., Dodson and Gillespie, 1967; Gill, 1989). These ideas are largely derived from the combined observations of patchy orchid distributions, small population sizes, and dust-like seeds that appear to have the potential to travel great distances and establish small, genetically isolated founder populations. One genetic expectation from this pattern of colonization would be a relatively high level of genetic variation distributed among populations [i.e., a high G_{ST} or the equivalent F_{ST} value]. This could be produced by repeated genetic bottlenecks during the colonization process. Based on the published isozyme studies of outbreeding orchid taxa reviewed in this study (Scacchi, Lanzara, and De Angelis, 1987; Scacchi and De Angelis, 1989; Scacchi, De Angelis, and Lanzara, 1990; Corrias et al., 1991; Scacchi, De Angelis, and Corbo, 1991; Peakall and Beattie, 1991; Rossi et al., 1992; Case, 1994; Arduino et al., 1995, 1996; Sun, 1996), there is no clear trend in G_{ST} values. In fact, G_{ST} values vary widely, ranging from less than 0.09 up to 0.75 with a grand mean of 0.159 [SE = 0.026; in a few cases, G_{ST} values in the above studies were recalculated to conform to the commonly reported methods of Nei (1973)]. This extensive variation in orchid G_{ST} values is not expected since the extent of among-population genetic variation is most highly correlated with the breeding system and life form of the plant (Hamrick and Godt, 1989). These factors would be highly similar for the taxa included in the above orchid studies. Another interesting result is that the grand mean G_{ST} value for the orchid studies cited above (this includes the means for the taxa in the present study, 0.163 and 0.182) is slightly smaller than averages reported by Hamrick and Godt (1989) for long-lived herbaceous perennials (0.213, SE = 0.144) or animal outcrossed taxa (0.197, SE = 0.017). This result suggests that, on average, orchids do not show higher levels of genetic differentiation among populations relative to other plants with similar life history characteristics. However, the large variance in G_{ST} values among orchid studies also indicates that the relative magnitude of a G_{ST} value for any specific orchid taxon is not easy to predict. This conclusion even applies to orchid taxa that appear nearly identical in their life history characteristics. The mean value of G_{ST} for the orchid taxa reviewed in the present study (calculated from 24 taxa) may be affected by the relatively low number of published isozyme studies in the Orchidaceae. Furthermore, there is a biased representation of some genera (e.g., *Orchis*) as well as a strong bias towards temperate species. For *Cypripedium* taxa in the present study, it is interesting to note that although genetic bottlenecks are apparently uncommon at the population level, significant bottlenecks involving *C. parviflorum* may have occurred on two occasions. This result suggests that genetic bottlenecks in this genus may be relatively rare, but that they may be associated with the formation of new taxa.

Although the major allele distribution does not indicate extensive among-population variation in *C. kentuckiense,* it is evident from this study that potentially significant geographic variation can exist among minor alleles. This especially applies to the differences found between the Virginia population of *C. kentuckiense* and the more western populations of this taxon. The Virginia population differs qualitatively from the western sites in three ways: (1) it lacks the high-frequency allele (*Got2-d*) that is present in all sampled western populations, (2) it contains an allele (*Got2-e*) that is present in all *C. parviflorum* var. *pubescens* populations but is not found in any other *C. kentuckiense* population, and (3) it contains a moderate-frequency, population-specific allele (*Pgm1-d*). Although there are several possible historical scenarios to explain these findings, one of the most parsimonious explanations is that there was an early eastern/western reduction of gene flow in *C. kentuckiense* prior to the evolution of *Got2-d* in the west and *Pgm1-d* in the east. The Virginia population would have also retained a putative ancestral allele *Got2-e* or acquired it through introgression with *C. parviflorum.* Because there was no evidence of possible introgression at other loci in the Virginia population and no *C. parviflorum* have been found near this population, the retention of an ancestral allele

from *C. parviflorum* var. *pubescens* appears to be the most likely explanation. To examine further the hypothesis of an early eastern/western split of *C. kentuckiense* populations, it will be necessary to locate and study additional populations of *C. kentuckiense* in the east. This additional data could be used to test the hypothesis that the Virginia population represents a remnant from a larger historical range for *C. kentuckiense* rather than a recent dispersal and colonization event from the west.

Given the relatively low levels of genetic variation in *C. kentuckiense* and the existence of populations with unique genetic variation (e.g., the Virginia population), we think it would be helpful for management efforts to attempt to maintain large population sizes and document existing threats to populations including biological and anthropogenic threats. These efforts will be especially important for any future re-evaluation of *C. kentuckiense* for the federal endangered and threatened species list. Lastly, the very high genetic and morphological similarity between *C. parviflorum* and *C. kentuckiense* might also be important for conservation efforts. Since manipulation of *C. kentuckiense* populations could endanger them, effective management techniques could be developed for *C. parviflorum,* which is more abundant and apparently less vulnerable to extirpation. The management techniques developed for *C. parviflorum* could be evaluated for transfer to *C. kentuckiense* populations.

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