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Genetic diversity of *Cypripedium calceolus* at the edge and in the centre of its range in Europe

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The patterns of genetic diversity in 14 *Cypripedium calceolus* populations were investigated in two distant regions, located inside its continuous range (Biebrza valley, NE Poland) and close to the periphery of its range (Alps, SE France). The genetic variation at the species level was found to be relatively high ($P\% = 37.5\%$, $H_O = 0.145$) as compared with that observed in other endangered or rare species. The mean genetic diversity for both European regions did not differ significantly, except for $P\%$ (42.5% for Biebrza valley, 34.1% for Alps, $p < 0.001$). The Biebrza valley yielded almost twice as many genotypes, but genotypic diversity (as measured by G/N and $\hat{G}/N$) was higher in populations from the Alps. This could mean that asexual reproduction is more intense in populations in the Biebrza valley than in the Alps. The results from PCA, UPGMA and AMOVA analyses showed clear genetic differences in *C. calceolus* between the two European regions. The Mantel test showed positive and significant associations between genetic and geographic distances among populations situated in the Biebrza valley ($r^2 = 0.37$, $p < 0.05$), but not among the Alpine populations ($r^2 = –0.25$, $p > 0.05$). Our data showed that substantial loss of genetic diversity in *C. calceolus* most likely occurs rather at the population than at the species level.

Key words: allozymes, conservation, genotypic diversity, geographic isolation, Lady's Slipper

Introduction

In the last few decades, studies employing molecular techniques have resolved many problems of plant genetic diversity and have yielded information important in many fields including evolution, ecology and conservation biology.

Abundant data on the genetic structure of plant populations have shed light on the regularities, causes and consequences of genetic variation (Loveless & Hamrick 1984, Hamrick & Godt 1989, Ellstrand & Ellam 1993, Karron 1997, Gitzendanner & Soltis 2000, Nybom 2004). The principal determinants of genetic variability in plant species are their biological properties (especially mating systems) and historical aspects connected with phylogeography (colonization processes; formation, origin and establishment of populations). Phenomena such as genetic drift, the founder effect, gene flow and natural succession are strictly connected with the latter aspect. In consequence, populations across a geographic range can exhibit different levels of genetic variation. The environmental conditions in which populations exist may also have an effect on patterns of population genetic structure. In various habitats, demographic processes also influence the genetic variation of plant populations, for example through differential levels of sexual reproduction or vegetative growth in clonal plants. One consequence of these relationships is variation of population size. In addition, when a habitat favours some genets they may dominate populations, significantly affecting the genetic structure (Murawski & Hamrick 1990, Brzosko & Wróblewska 2003).

In species with wide geographic ranges, one can expect populations from different parts of the distribution range to have distinct genetic structures. There is also evidence that both the ecological and genetic structures of populations are shaped differently in continuous than in discontinuous ranges (Furnier & Adams 1986, Tremblay & Simon 1989, Lagercrantz & Ryman 1990, Persson *et al*. 1998, Lammi *et al*. 1999, Reinhammar 1999, Després *et al*. 2002, Tyler 2002, Schönswetter *et al*. 2003). In other words, the distribution of populations in space dictates the rates of gene flow among them and affects the spatial variation of their genetic composition (Frankham *et al*. 2003). In studies of genetic diversity across species' geographic ranges, peripheral populations seem to be more interesting than central ones as subjects for analysis. Theoretical and empirical studies have confirmed that marginal populations are more sensitive to genetic drift and/or strong directional selection (Barrett & Husband 1990). Genetic diversity should be depleted within small and repeatedly isolated populations located at the edge of a range (Furnier & Adams 1986, Hannan & Orich 2000, Langergott *et al*. 2000, Johes *et al*. 2001). Isolation resulting from geographic barriers (mountains, rivers, deserts, etc.) is manifested on wider geographic scales. Isolation also results from habitat fragmentation due to human activity or intense natural succession within still-open habitats. Fragmentation is one of main factors behind the occurrence of small populations (Lande 1988, Ellstrand & Elam 1993, Morris 1993, Lennartsson 2002). The most common view is that large populations typically contain higher levels of genetic variation and show negligible changes in genetic diversity over time, while small populations become genetically impoverished and rapidly lose genetic diversity.

We investigated genetic differentiation among the populations of the orchid *Cypripedium calceolus* (Lady's Slipper) in the context of the above questions in two European regions differing in many respects: (1) the Biebrza valley in NE Poland and (2) different mountain massifs in the French Alps. The aims of our investigations were: (1) to assess the levels of genetic variation of *C. calceolus* populations in relation to life history characteristics in two very distant European regions. Such data also allow rough estimates of genetic variation at the species level in European populations of *C. calceolus*, (2) to compare genetic differentiation between *C. calceolus* populations within a relatively small region such as the Biebrza valley (centre of the distribution range), on a wider scale among populations in different Alpine mountain massifs (periphery of the range), and between these two distant regions.

Material and methods

The study object

Cypripedium calceolus is found across Eurasia from Great Britain to southern Siberia. In the western part of its range the species extends northward to the Arctic Circle in Fennoscandia and southward to the Pyrenees, the Alpine Arc

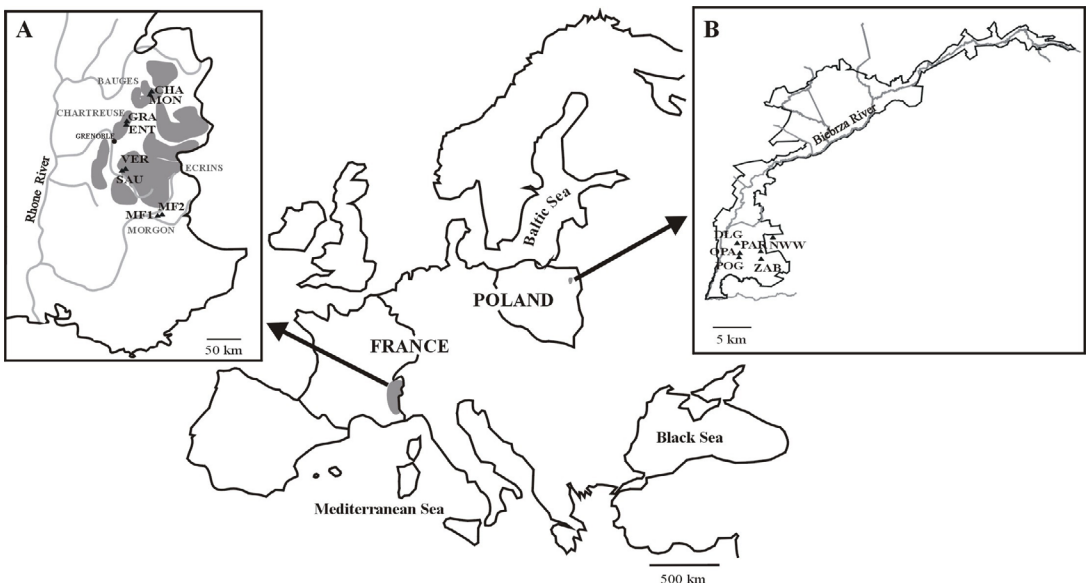

Fig. 1. Location of *Cypripedium calceolus* populations analysed: (**A**) the Alps and (**B**) the Biebrza valley (shaded area = location of the studied populations; the triangles – studied populations). Site codes of populations are indicated in Table 1.

and the Carpathians (Terschuren 1999). It is one of the most endangered plant species in Europe (Kull 1999). It is a herbaceous perennial, which reproduces both sexually and by vegetative propagation. *Cypripedium calceolus* is assumed to be an outcrosser, pollinated by small bees with highly restricted ranges. It may also produce new ramets from a horizontal rhizome underground. *Cypripedium calceolus* is a mainly boreal species which occurs in shady deciduous and mixed woodland (rarely in full sunlight), predominantly on calcareous soils (Kull 1999).

Study areas and plant material

The study areas were situated in two different geographic regions: (1) NE Poland (Biebrza valley, hereafter called Biebrza) and (2) SE France (Alps; Fig. 1). These two regions differ considerably with respect to topography, environmental heterogeneity and spatial structure. The Biebrza populations are situated in the centre of the species range, in a single protected area of the Biebrza National Park, and are very close to each other (0.25–6.7 km apart, average distance 3.5 km). The samples were collected from six different sites (mineral islands) among wetlands (Table 1). There are several mineral islands among the peat bogs of Biebrza, and *C. calceolus* populations occur at only six of them. All known populations in the studied region were analyzed, each from a given mineral island (Fig. 1). The *C. calceolus* populations in Biebrza National Park seem to differ considerably with respect to human impacts. Population NWW is very close to farmland and a regular road, and population PAR is near a tourist trail. The other four populations are on mineral islands fairly distant from any roads or paths, and are thus relatively less subject to anthropopression. Samples from three populations (ZAB, POG, OPA) have been studied previously (Brzosko *et al.* 2002a, 2002b); we included them in this analysis to obtain a wider picture of genetic variation in *C. calceolus* from this region. The Biebrza populations were of different sizes. The largest was DLG, which in the year of maximum abundance consisted of more than 2000 shoots (Brzosko 2002). The smallest population, NWW, consisted of only 28 shoots, all of which were sampled (Table 1). Plant material was collected from between 28 and 290 shoots from each of the Biebrza populations (Table 1). In the French Alps,

Table 1. Characteristics of *Cypripedium calceolus* populations within Biebrza and Alp regions; number of observed (N) and the total number of studied shoots (N_s), mean number of alleles per locus (A), percentage of polymorphic loci (P%), observed (H_O) and expected heterozygosity (H_E), the inbreeding coefficient for each population (F_{IS}) and the exact test probability associated with the Hardy-Weinberg test, number of genotypes (G), clonal diversity (G/N), Simpson's diversity index (D), number of unique genotypes (G_U), percentage of unique genotype ($G_{U\%}$), # sum of unique genotypes in regions and on the species level, Stoddart's and Taylor's genotypic diversity index ($\hat{G}$), normalized value of genotypic diversity index ($\hat{G}/N$). Genetic and genotypic diversity parameters from ZAB, OPA and POG populations (Biebrza valley) were included from Brzosko *et al.* (2002b). * $p < 0.001$.

Population	Code	N	N_s	A	P%	H_O	H_E	F_{IS}	G	G/N	D	G_U ($G_{U\%}$)	$\hat{G}$	$\hat{G}/N$ (%)
Biebrza valley (NE Poland)														
Nowa Wieś	NWW	28	28	1.45	27.3	0.153	0.119	–0.256	11	0.39	0.90	2 (18.2)	7.4	26.5
Parking	PAR	239	128	1.52	45.5	0.162	0.156	–0.025	31	0.24	0.88	7 (22.6)	8.0	6.2
Długi Grąd	DŁG	2000	390	1.72	45.5	0.164	0.176	0.089	75	0.19	0.95	23 (30.7)	7.9	2.0
Zabudnik	ZAB	1000	290	1.73	45.5	0.111	0.179	0.361*	43	0.15	0.94	14 (32.6)	11.1	3.8
Oparzelisko	OPA	600	218	1.73	45.5	0.178	0.181	0.056	55	0.25	0.98	19 (34.5)	15.5	7.1
Pogorzały	POG	600	202	1.73	45.5	0.166	0.175	0.048	56	0.27	0.97	14 (25.0)	19.0	9.4
Total				1.64	42.5	0.155	0.164	0.045	38	0.22	0.94	79# (49.6)	11.5	9.1
Alps (SE France)														
Bauges														
Montlardier	MON	796	57	1.55	36.4	0.132	0.142	0.058	30	0.53	0.97	2 (6.7)	19.8	14.0
Chapelle de Bellevaux	CHA	564	52	1.45	27.3	0.099	0.130	0.253*	24	0.46	0.96	2 (8.3)	18.3	35.0
Total				1.50	31.8	0.115	0.136	0.155	27	0.49	0.96	4# (8.9)	19.1	24.5
Chartreuse														
Entremont	ENT	1588	141	1.72	45.5	0.141	0.151	0.119	46	0.33	0.96	4 (8.7)	20.5	35.9
Granier	GRA	488	53	1.57	36.4	0.143	0.149	0.105	32	0.60	0.97	3 (9.4)	8.5	20.2
Total				1.64	40.9	0.142	0.150	0.112	39	0.46	0.96	7# (12.0)	14.7	28.1
Ecrins														
Verneys	VER	63	42	1.45	36.4	0.170	0.151	0.012	18	0.43	0.90	1 (5.6)	13.4	39.5
Sauzerie	SAU	268	49	1.36	27.3	0.112	0.120	–0.002	19	0.39	0.94	0 (0.0)	13.8	29.9
Total				1.41	31.8	0.141	0.135	0.005	18	0.41	0.92	1# (3.2)	13.6	34.7
Morgon														
Boscodon Forest 1	MF1	700	34	1.45	27.3	0.166	0.141	–0.072	18	0.53	0.95	0 (0.0)	13.0	26.5
Boscodon Forest 2	MF2	425	46	1.51	34.1	0.134	0.137	0.101	20	0.44	0.95	1 (5.0)	22.8	43.0
Total				1.48	30.7	0.150	0.139	0.015	19	0.48	0.95	1 (3.2)	17.9	34.8
Total				1.51	33.8	0.137	0.140	0.072	26	0.46	0.95	13# (14.9)	16.3	30.5
Species level				1.56	37.5	0.145	0.150	0.041	31	0.37	0.94	92# (49.5)	14.2	21.4

the species occurs in mountain habitat. Samples were collected from two sampling sites on each of four different mountain massifs (Bauges, Chartreuse, Écrins, Morgon; Fig. 1 and Table 1). The distance between those sampling sites ranged from 0.5 to 135 km (average 70 km). All French *C. calceolus* populations were situated in silver fir-beech forests at 1000–1400 m a.s.l. on slopes ranging from 10° to 55°. Population size varied from 63 to 1600 shoots. The study sites in Bauges, Chartreuse and Écrins were in protected areas (Massif des Bauges Regional Park, Chartreuse Regional Park, Écrins National Park). In the Morgon massif, the sites in the Boscodon forest are within a Natura 2000 protected area. All the sites are very attractive for tourism and are crossed by many hiking paths, forest tracks and small roads. Populations MON, CHA and ENT are not visible from any of the paths and are not easily accessible because they are on very steep slopes. Part of population MF1 is visible from a small forest road. Populations VER and GRA are near farmland, and GRA is along a track used by hikers and tractors. Population MF2 is near a hiking path and a picnic area.

Isozyme electrophoresis and genetic analyses

The number of *C. calceolus* samples from Biebrza and the Alps used for the study was 1595 (1121 for Biebrza, 474 for the Alps; Table 1). For each ramet sampled, one fresh leaf tip (approximately 2 cm long) was removed and frozen in liquid nitrogen, and later the samples were ground and analyzed by protein electrophoresis. Ten enzyme systems encoding eleven loci (*Adh*, *Est*, *Gdh*, *Got*, *Idh-1*, *Idh-2*, *Mdh*, *6Pgd*, *Pgi*, *Pgm*, *Skd*) were analyzed by electrophoresis on 12% starch gels using two buffer systems according to protocols given by Brzosko *et al.* (2002a, 2002b).

Cypripedium calceolus reproduces both sexually and vegetatively, and usually occurs in clearly distinguishable clumps (aggregations of shoots) within populations. We took the samples either from a single shoot or from all shoots in a given clump. All sampled shoots were sorted by multilocus genotype based on polymorphic loci. To study genetic variation parameters, we included only one repeated genotype belonging to a given genet from a given clump (individual in the genetic sense). The majority of samples were collected as single shoots from different clumps. Samples collected in this way did not allow for assessment of clonal diversity; for that, all sampled shoots collected from a given genet were taken into account in clumps where several samples were taken.

Measures of genetic variation were calculated for each population using GENEPOP ver. 3.2 (Raymond & Rousset 1995) and FSTAT ver. 2.9.3 (Goudet 2001). The following measurements were obtained: mean number of alleles per locus (A), percentage of polymorphic loci ($P\%$; a locus was considered polymorphic if more than one allele was detected), and average observed (H_O) and expected (H_E) heterozygosity values. Multilocus F_{IS} values were also obtained for each sample and tested by 1000 permutations using FSTAT. The sequential Bonferroni technique was employed for significance testing (Rice 1989). The Biebrza and Alpine populations were compared with respect to $P\%$, A, H_O and F_{IS} using a Mann-Whitney U-tests. Relationships between parameters of genetic variation such as $P\%$ and H_O and population size were examined with Spearman's rank correlations (r_S) using StatSoft ver. 6.0 (StatSoft Inc. 2001). Deviations from Hardy-Weinberg equilibrium were tested for each locus and in every population, using GENEPOP. A global probability value over all loci was obtained using Fisher's method (Sokal & Rohlf 1995). Tests for linkage disequilibrium for all locus pairs within each population were performed using GENEPOP. To test for recent reduction of effective population size, BOTTLENECK ver. 1.2.02 (Cornuet & Luikart 1997) was used. Because alleles are generally lost faster than heterozygosity (Hedrick *et al.* 1986), recently bottlenecked populations will display excess heterozygosity relative to that expected from the number of alleles.

Each of the detected distinct multilocus genotypes was assumed to be a distinct genet. We counted genotypes present exclusively within each region, genotypes common to all populations, and genotypes unique to a single population within a given region. Two different meas-

ures of clonal diversity were calculated in the study. The first was G/N, where G is the number of genotypes and N is the number of shoots sampled. G/N is the probability that the next shoot sampled will be a different genotype. The second measure of genet diversity is Simpson's index (D) corrected for finite sample size (Pielou 1969). Genotypic diversity in each population was also calculated using Stodart and Taylor's index (1988): $\hat{G} = 1/\Sigma f_x(x/N)^2$, where f_x is the number of genotypes observed x times in the sample, x is the number of times the genotype is observed, and N is the sample size. Because the two populations had different sample sizes, $\hat{G}$ was divided by N to normalize the diversity measure.

To compare the amount of genetic variation partitioned within and among populations, F-statistics according to Weir and Cockerham (1984) were estimated over all loci for both regions separately, using GENEPOP. The 95% confidence intervals (CI) for overall F_{ST} were obtained by the bootstrapping procedure (1000 iterations) in FSTAT. The significance of all values was ascertained by 1000 permutations, not assuming HWE within samples. Mantel tests were conducted to examine the relationship between pairwise $F_{ST}/(1 - F_{ST})$ and log geographic distances between sampling sites (Biebrza and Alpine populations analyzed separately) using GENEPOP.

Two different hierarchical analyses of molecular variance AMOVA were performed to study the genetic structure of *C. calceolus* populations with ARLEQUIN ver. 2.0 (Schneider *et al.* 2000). First, AMOVA was used to estimate the partitioning of the total genetic diversity between regions: the Biebrza valley and the Alps. Then another AMOVA was computed with division of the Alpine populations according to their geographic locations, that is, according to their location in four different mountain massifs. Levels of significance for populations were determined using the permutation test (1000 permutations) to obtain solid test statistics.

Evidence of group distinctness was obtained using Principal Component Analysis (PCA) of allozyme gene frequency data. This was done to investigate the spatial patterns of genetic variation. For this, PCAGEN ver. 1.2 (Goudet 2001) was used. In addition, Nei's (1978) unbiased genetic distance was calculated among the populations studied. Finally, we constructed a UPGMA tree using TFPGA ver. 1.3 (Miller 1997). Branches important for discerning host-associated populations/groups were evaluated by a bootstrap approach with 1000 iterations. Only branches with bootstrap support above 70% were taken into consideration (Felsenstein 2004).

Results

Genetic diversity at species and population levels

Analysis of 1595 *C. calceolus* samples from 14 populations revealed five polymorphic isozyme loci (*Got*, *Idh-1*, *Idh-2*, *6Pgd*, *Pgm*). Allele frequencies for these loci are presented in the Appendix. The number of alleles at variable loci was two, except for the *Got* locus which was characterized by the presence of five alleles. The remaining six loci (*Adh*, *Est*, *Gdh*, *Mdh*, *Pgi*, *Skd*) were monomorphic in all populations. A total of 19 alleles were recorded at 11 loci, with a mean of 1.73 alleles per locus for the total sample. None of the alleles were unique to any population or geographic region (Appendix).

The mean values of genetic variation parameters at the species level were relatively high ($P\%$ = 37.5%, H_O = 0.145). These values differed between regions and among populations. There were significant differences in $P\%$ values between the two regions ($P\%$ = 42.5% for Biebrza *vs.* 33.8% for Alps, $U = 5.0$, $p = 0.014$); the values of A and H_O were higher in Biebrza than in the Alps but not significantly ($A = 1.64$ *vs.* 1.51, $U = 9.0$, $p = 0.053$, $H_O = 0.155$ *vs.* 0.137, $U = 14.5$, $p = 0.220$). Similarly, F_{IS} did not differ between the Biebrza and Alpine sampling sites (0.045 *vs.* 0.071, $U = 19.0$, $p = 0.518$). The genetic variation parameters varied considerably at the population level. The average number of alleles (A) per locus within populations ranged from 1.36 (SAU, Alps) to 1.73 (ZAB, OPA, POG, Biebrza valley), and the proportion of polymorphic loci (P) ranged from 27.3% (MF1, Alps) to 45.5% (ZAB, OPA, POG, Biebrza valley). The observed heterozygosity values ranged from 0.099 (CHA, Alps) to 0.178 (OPA, Biebrza valley; Table 1). The F_{IS}

values were also highly variable, particularly between the Biebrza populations, and ranged from –0.256 (NWW) to 0.361 (ZAB, significant heterozygote deficit; Table 1). The *P%* and H_O values were not significantly correlated with population size (Spearman's rank correlation: $r_S = 0.29$ for *P%*, $r_S = –0.10$ for H_O, $p > 0.05$) for the whole data set.

There were significant deviations from Hardy-Weinberg equilibrium for 1–3 loci and in global probability tests within seven populations. HWE was not found for the *Got* locus in five populations, nor for the *Pgd*, *Pgm* and *Idh-1* loci in two populations, suggesting a departure from random mating in these populations. The *Idh-2* locus was variable in six populations only, and it was always in HWE. No significant (Bonferroni-corrected) pairwise linkage disequilibria between pairs of loci were detected within any of the populations studied. Wilcoxon tests for genetic signatures of a population bottleneck using the BOTTLENECK program did not reveal any evidence of recent reduction in size in any of the populations studied ($p < 0.05$).

Clonal diversity

Using five polymorphic loci, we identified 186 different multilocus genotypes in 14 *C. calceolus* populations. We found almost twice as many genotypes (159) in the six populations from the Biebrza valley than in the eight Alpine populations (87). We found 99 genotypes present exclusively in the Biebrza samples, while only 28 were present exclusively in the Alps. The remaining 59 genotypes were common to both groups of populations. No genotype was common to all populations studied; 22 genotypes (11.8%) were observed in at least 50% of the populations. Half of all genotypes (92) were singletons. The number of unique genotypes counted for a given population was higher for Biebrza (79) than for the Alps (13). The populations from Biebrza had 2–23 unique multilocus genotypes. In two populations from the Alps such genotypes were absent, and in the others the number of unique genotypes ranged from 1 to 4 (Table 1). It should be noted, however, that the probability of finding a new genet (*G*/*N*) in the Alps (average *G*/*N* 0.46) was almost double that for the populations from Biebrza (0.25). Clonal diversity (*D*) ranged from 0.88 in PAR to 0.98 in OPA (both from Biebrza valley; Table 1). Stoddart and Taylor's (1988) average genotypic diversity ($\hat{G}$) in the Alpine region was 16.3 (range = 8.5–22.8), while in the Biebrza region it was 11.5 (range = 7.4–19.0). The values of $\hat{G}/N$ correcting $\hat{G}$ for sample size differences were several times higher in the Alpine (average $\hat{G}/N$ = 30.5%, range = 14.0%–43.0%) than in Biebrza populations (average $\hat{G}/N$ = 9.1%, range = 2.0%–26.5%).

Genetic differentiation among populations

Overall genetic differentiation in both the Biebrza and Alpine groups was low, albeit significant (Biebrza: $F_{ST} = 0.041$, 95% CI = 0.012–0.077, $p < 0.001$; Alps: $F_{ST} = 0.053$, 95% CI = 0.043–0.063, $p < 0.01$). The pairwise F_{ST} values calculated between populations within a given region varied widely, ranging from 0.003 to 0.156 (Alps) and from 0.002 to 0.275 (Biebrza valley) (Table 2). The corresponding probabilities indicated significant population differentiation for 71% of the population pairs in the Alpine group and for 87% of the pairs in the Biebrza group. The most divergent population pairs were NWW and PAR in the Biebrza valley and MF2 and SAU in the Alps (Table 2). A significant pattern of isolation by distance was found in Biebrza: $F_{ST}/(1 - F_{ST}) = 0.0009 + 0.0862\log(\text{distance})$, $r^2 = 0.37$, $p < 0.05$). In contrast, there were no IBD patterns in the Alpine group: $F_{ST}/(1 - F_{ST}) = 0.104 - 0.011\log(\text{distance})$, $r^2 = –0.25$, $p > 0.05$; geographically close populations were not necessarily genetically similar. For example, populations MF1 and MF2 were separated at distance about 4 km and were on the same mountain massif (Boscodon), but the corresponding pairwise F_{ST} value was one of the highest (0.157).

Analysis of molecular variance (AMOVA) for *C. calceolus* showed low though significant differentiation between the Biebrza valley and the Alps ($F_{CT} = 0.027\%$, $p < 0.001$). Most of the variation was found within populations (92.45%,

$p < 0.001$), and 4.77% of the total variation was partitioned between samples within region (Table 3). Another AMOVA with division into four mountain massifs in the Alps explained only 0.22% of the total variation and was not statistically significant ($p = 0.363$; Table 3). Principal component analysis (PCA) based on all populations sampled generated two principal components, in which the first PC axis explained 40.7% ($p = 0.388$) and the second axis (PC2) 31.5% ($p = 0.027$) of the total genetic diversity; it also showed separation between the Biebrza and Alpine regions to a certain extent (Fig. 2a). The two smallest populations from Biebrza were closer to the French samples with respect to multilocus genotypes. The way the populations tended to cluster within a given region was independent of their geographic position in the whole data set. Moreover, geographically close populations did not cluster together in any case. The ordination diagrams for within-region genetic structure also showed distinct patterns for both the Biebrza valley and the Alps. In the Biebrza valley, populations formed a few separate

Table 2. Genetic differentiation F_{ST} (below diagonal) and p values ascertained by 1000 permutations (above diagonal) between pairs of the Biebrza and Alpine populations. ** $p < 0.01$, *** $p < 0.001$, NS = not significant.

Biebrza valley	NWW	PAR	DŁG	ZAB	OPA	POG
NWW	–	***	***	***	***	***
PAR	0.275	–	***	***	***	***
DŁG	0.145	0.030	–	***	***	NS
ZAB	0.182	0.082	0.023	–	**	NS
OPA	0.233	0.039	0.024	0.016	–	***
POG	0.162	0.043	0.002	0.012	0.020	–

Alps	Bauges		Chartreuse		Ecrins		Morgon	
	MON	CHA	ENT	GRA	VER	SAU	MF1	MF2
MON	–	***	NS	***	NS	***	NS	***
CHA	0.068	–	***	***	***	***	NS	***
ENT	0.006	0.067	–	NS	NS	***	NS	***
GRA	0.025	0.082	0.005	–	***	***	***	***
VER	0.013	0.073	0.003	0.021	–	***	NS	***
SAU	0.066	0.117	0.045	0.042	0.069	–	***	***
MF1	0.009	0.042	0.008	0.016	0.022	0.058	–	***
MF2	0.156	0.094	0.103	0.133	0.117	0.111	0.157	–

Table 3. Hierarchical AMOVA of five allozyme loci in *Cypripedium calceolus* populations.

Source of variation	Variance components	Percentage of variation	p
Biebrza valley and the Alp regions			
Among Biebrza and Alpine regions	0.027	2.78	0.001
Among populations within regions	0.046	4.77	0.001
Within populations	0.883	92.45	0.001
$F_{CT} = 0.027$			0.001
$F_{ST} = 0.075$			0.001
$F_{SC} = 0.049$			0.001
Four massifs of the Alps			
Among Alpine massifs	0.002	0.22	0.363
Among populations within massifs	0.048	5.56	0.001
Within populations	0.883	94.22	0.001
$F_{CT} = 0.002$			0.363
$F_{ST} = 0.056$			0.001
$F_{SC} = 0.058$			0.001

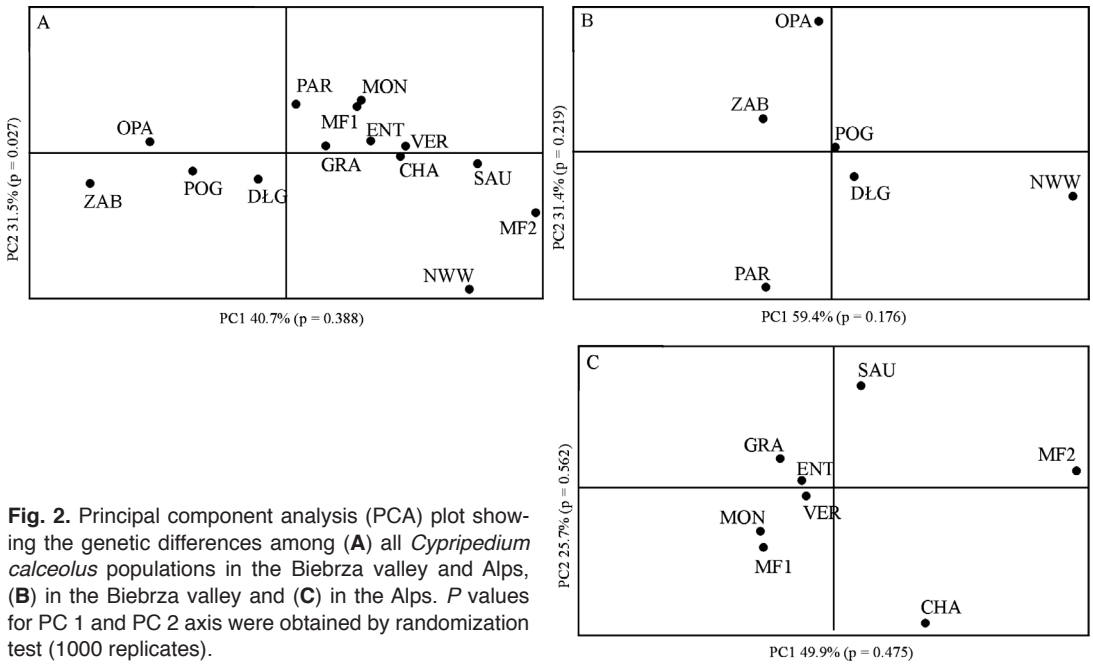

Fig. 2. Principal component analysis (PCA) plot showing the genetic differences among (**A**) all *Cypripedium calceolus* populations in the Biebrza valley and Alps, (**B**) in the Biebrza valley and (**C**) in the Alps. *P* values for PC 1 and PC 2 axis were obtained by randomization test (1000 replicates).

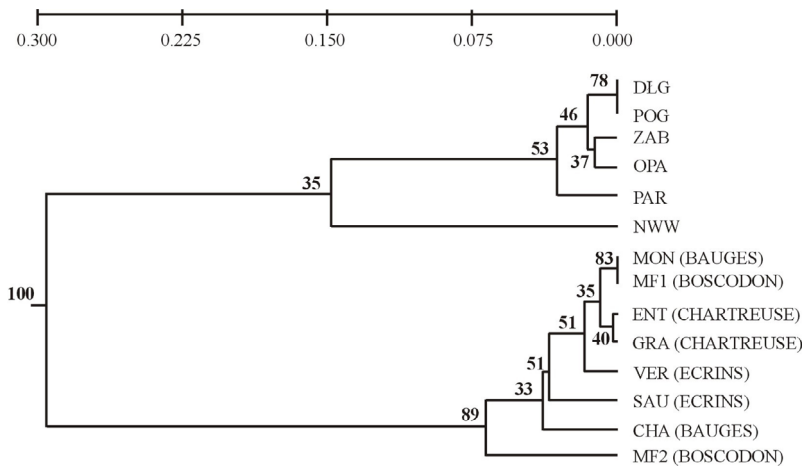

Fig. 3. UPGMA tree based on Nei's genetic distance among *Cypripedium calceolus* populations from both European regions; in the brackets are written the name of the Alpine massifs. Bootstrap values (1000 replicates) are present for each node.

groups on PC plots (59.4%, $p = 0.176$, Fig. 2b). The population pairs from each massif located in the Alps did not cluster together on the diagram illustrating PC plots with respect to their geographic position (PC1 = 49.9%, $p = 0.475$), except for ENT and GRA (Fig. 2c).

UPGMA analysis based on Nei's (1978) genetic distance supported the results of PCA analysis for the whole data set (Fig. 3). The differences between the two distinct European regions were highly supported (100% of bootstrap). Populations DLU and POG (78% of bootstrap) as well as ZAB and OPA (37% of bootstrap) from the Biebrza valley clustered together on the two diagrams (Fig. 3). In the case of the Alpine populations, only ENT and GRA grouped together according to their location in the Chartreuse massif, but the node had fairly low bootstrap support (40%, Fig. 3). Populations MON and MF1 from different mountain massifs grouped together (83% of bootstrap), and MF2 was distinct from the others (89% of bootstrap).

Discussion

Genetic diversity at species and population levels

The level of genetic diversity is strictly connected with the spatial scale. Often, small fragments of the total geographic range of a species are studied. We should be aware that knowledge about genetic diversity based on small samples or regions may not carry over to the entire species. Extensive knowledge of genetic structure is possible when investigations include population samples from the whole range of a given species. In the case of endangered species we observe only the current genetic variation, which in many cases is a small fragment of the species variation that existed before the population underwent a drastic reduction in number and size.

Despite increasing the total sample size by 11 new populations of *C. calceolus*, we found almost the same levels of genetic variation at the species level as in the previous study of three Polish populations ($P\% = 45.5\%$, $A = 1.73$; Brzosko *et al.* 2002a, 2002b). Therefore, we can assume that the degree of genetic diversity at the species level, as measured by conventional parameters ($P\%$ and A), may be characteristic for *C. calceolus* at least in the European part of its range. Analyses at the level of genotypic diversity, however, showed that the number of distinct multilocus genotypes increased considerably (from 109 to 186) with the increased number of samples.

The total level of genetic variation over all 14 *C. calceolus* populations studied is higher than for other endangered or rare species as summarized by Hamrick and Godt (1989). It may vary widely among species belonging to the genus *Cypripedium*; for example, the percentage of polymorphic loci ($P\%$) ranges from 0% to 81.8% (Bornbusch *et al.* 1994, Case 1994, Case *et al.* 1998, Wallace & Case 2000).

Exclusive outcrossing coupled with extensive vegetative reproduction seem to be a major factor promoting the relatively high genetic diversity in *C. calceolus* (Kull 1999, Brzosko *et al.* 2002a, 2002b). In the case of *C. calceolus* from the Biebrza valley, monitored for 18 years, it has been suggested that genetic diversity may have been maintained by regular recruitment of new individuals or intense vegetative reproduction (Brzosko 2002, Brzosko *et al.* 2002a, 2002b). On the other hand, special aspects of its biology, such as high specialization with respect to pollinator behaviour (small bees with a highly restricted area), habitat (calcareous and wet), obligatory mycorrhizal infection during the entire life cycle, and prolonged development of the protocorm, may lower levels of genetic variation. In both of the populations studied, the most important attribute affecting genetic variation of the species is isolation in time and space due to their location in different areas of the range. The Alpine populations are close to the margin of its continuous range and are distributed in different mountain massifs, while the Biebrza populations occur on mineral islands separated by peat bogs in the centre of the range. On the other hand, the Biebrza and Alpine populations are similar with respect to anthropopression, which slightly alters and damages the natural environment, particularly in two populations in the Biebrza valley (NWW, PAR) and three populations in the Alps (VER, MF2, GRA). Nevertheless, in both of these European regions the mean genetic diversity is maintained on similar levels and differs non-significantly, except for differences in $P\%$. Of course this does not mean that the populations from the two regions are very similar. Differences between the regions do exist, as revealed by $P\%$, and also involve the patterns of genotypic diversity as assessed from the total number of detected genotypes, the genotypes exclusive to each region, and the number of genotypes unique to populations. In the Biebrza valley we found almost twice as many genotypes, but genotypic diversity as measured by G/N and $\hat{G}/N$ was higher in the populations from the Alps. This could mean that asexual reproduction is more intense in the populations from Biebrza than in the Alps. Note, however, that there were three times as many exclusive genotypes in the Biebrza populations as in the Alps. One possible cause of the differences in genotypic structure between the two regions and among populations is restricted seed dispersal. The commonly held view that orchid seeds are disseminated by long-distance dispersal is challenged by new data reported by Machon *et al.*

(2003), indicating that seed dispersal in *Spiranthes spiralis* is limited to the neighbourhood of the fruiting plants. This was also confirmed by our preliminary experiment in the field on *C. calceolus*, *Epipactis helleborine* and *Goodyera repens* populations, in which seed dispersal occurred mostly at short distances, within the margins of fruiting ramets or around them (E. Brzosko unpubl. data). Thus, within-population genotypic diversity is enriched mainly through the appearance of new individuals from the local seed bank, and the probability of new genets arriving from other populations is very low. Peripheral populations are likely to have been colonized or recolonized more recently than central ones (Lönn & Prentice 2002); this may account for the lower number of multilocus genotypes in the Alpine populations.

We did not find statistically significant correlations between population size and the level of genetic variation. This result is in agreement with previous studies of clonal plants (with two types of reproduction), where population size based on the number of ramets was not significantly correlated with intra-population genetic diversity (Lannér-Herrera *et al*. 1996, Schmidt & Jensen 2000, Tero *et al*. 2003). It should be noted, however, that smaller populations were usually characterized by lower values of such parameters as *P%*, *A* and H_O in those studies. In the present study of the Alpine populations, only the biggest one (> 1500 shoots) had maximal values of *P%* and *A*. We suggest that a conspicuous decrease of genetic diversity occurs below some threshold value of population size. The situation is especially clear for the smallest NWW population in the Biebrza valley, which had the lowest genetic and genotypic diversity. Although we did not detect the effect of a bottleneck in the smallest population (NWW) from Biebrza, situated near farmland, we know that the population declined in recent times (personal information from Biebrza farmers who observed this population for decades). The low level of variation could very well threaten the persistence of this population. In the Alpine populations, there could be other factors shaping genetic diversity, for example colonization events and genetic drift.

Geographic isolation and dispersal

The PCA, UPGMA and AMOVA results brought out evident genetic differences between the two European regions within *C. calceolus*. The most striking results were from UPGMA analysis, which grouped the populations from the Biebrza valley and Alps into separate clusters with 100% bootstrap support. Ongoing and significant processes of isolation between these distant regions were illustrated by AMOVA ($F_{CT} = 0.027$, $p < 0.001$). We also obtained low but statistically significant F_{ST} values within the Biebrza valley ($F_{ST} = 0.041$, $p < 0.001$) and Alps ($F_{ST} = 0.053$, $p < 0.01$), but found a positive and significant association between genetic and geographic distances for the Biebrza valley only. We suggest that the relatively low differentiation of *C. calceolus* populations in the Biebrza valley can be explained by significant historical levels of the gene flow when the populations were more connected, and/or by current gene flow following the stepping stone model.

Brzosko *et al*. (2002a) suggested that the Biebrza populations may have originated from a single source of colonizers. This seems supported by AMOVA: only 4.77% of genetic variation was among *C. calceolus* populations within regions, and more than 90% within populations. Other studies support the general observation that long-lived, outbreeding and wind-dispersed species maintain most of their variation within populations (Gugerli *et al*. 1999, Stehlik *et al*. 2001).

Contrary to our expectations, no relationship between genetic and geographic distances and no hierarchical structuring was found among populations located on different mountain massifs within the Alps ($F_{CT} = 0.002$, $p = 0.363$), and this was confirmed by PCA and UPGMA analyses. Klimes *et al*. (1997) and Gaudeul *et al*. (2000) noted that Alpine habitats often are characterized by extreme patchiness and strong natural fragmentation. Thus, spatial isolation of Alpine populations is probably more the rule than the exception. Because of the heterogeneity of the landscape, Alpine species are organized into local populations of different sizes and are highly structured spatially. The non-significant relationship between genetic and geographic dis-

tance and the lack of a spatial pattern among the Alpine *C. calceolus* populations in adjacent massifs suggest that they were not necessarily founded from propagules of the nearest or older populations from the same area, but by immigrants from more distant places. In another Alpine plant species, *Eryngium alpinum*, Gaudeul *et al.* (2000) observed random genetic differentiation among populations, even due to occasional long-distance colonization events out of a single periglacial refuge, and they explained the lack of clear spatial genetic structure by extreme bottlenecks in *E. alpinum* populations; we did not confirm this phenomenon in the Alpine *C. calceolus* populations.

Conservation implications

The central problem for conservation genetics is identification of management units (MUs) and evolutionarily significant units (ESUs) (Moritz 1994). Although the methods used in this study are not designed to distinguish such units according to the rules given in the above papers, we stress that all the *C. calceolus* populations we studied should have special conservation status. Each of them is a more or less genetically distinct structure which contributes to the total genetic diversity of this species. This is best illustrated by the findings that no genotype was common to all 14 populations and that half of the genotypes were unique to a given population. Thus, each of them represents a unique gene combination comprising a distinct fragment of biodiversity. For species requiring conservation such as *C. calceolus*, each component of its genetic diversity is critical to preventing its extinction. Our data suggest that substantial loss of genetic diversity occurs more rapidly at the population than at the species level. The genetic erosion in a few populations of *C. calceolus* suggests that conservation efforts should focus below the species or even regional level to stem the further decline of its genetic resources and to support its evolutionary potential. Conservation efforts should take account of all knowledge about *C. calceolus*: its biology, environmental and demographic observations of populations, and data on their genetic and genotypic diversity. This information should be combined to achieve a more precise view of the status of the species.

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Appendix. Allele frequencies in *Cypripedium calceolus* populations located in Biebrza valley and Alps. For explanation of population codes *see* Table 1. The values of allele frequency in ZAB, OPA and POG populations are also included in Brzosko *et al.* (2002b).

		Biebrza valley (NE Poland)						Alps (SE France), Massifs						
								Bauges		Chartreuse		Ecrins		Morgon	
Locus	Alleles	NWW	PAR	DŁG	ZAB	OPA	POG	MON	CHA	ENT	GRA	VER	SAU	MF1	MF2
Got	*a*	0.219	0.087	0.073	0.018	0.029	0.053	0.000	0.000	0.068	0.130	0.000	0.000	0.000	0.000
	b	0.063	0.348	0.174	0.165	0.159	0.143	0.386	0.200	0.369	0.210	0.294	0.139	0.457	0.280
	c	0.000	0.000	0.045	0.024	0.071	0.043	0.080	0.329	0.011	0.000	0.044	0.000	0.109	0.140
	d	0.500	0.359	0.330	0.193	0.306	0.271	0.420	0.286	0.398	0.400	0.485	0.736	0.348	0.420
	e	0.219	0.207	0.378	0.600	0.435	0.490	0.114	0.186	0.153	0.260	0.176	0.125	0.087	0.160
Pgm	*a*	0.563	0.609	0.601	0.600	0.694	0.575	0.659	0.886	0.636	0.580	0.941	0.667	0.587	0.680
	b	0.438	0.391	0.399	0.400	0.306	0.425	0.341	0.114	0.364	0.420	0.059	0.333	0.413	0.320
Pgd	*a*	0.094	0.011	0.094	0.206	0.194	0.101	0.045	0.000	0.028	0.010	0.059	0.000	0.022	0.000
	b	0.906	0.989	0.906	0.794	0.806	0.899	0.955	1.000	0.972	0.990	0.941	1.000	0.978	1.000
Idh-1	*a*	0.000	0.761	0.521	0.82	0.706	0.559	0.716	0.571	0.580	0.590	0.265	0.514	0.522	0.720
	b	1.000	0.239	0.479	0.418	0.294	0.441	0.284	0.429	0.420	0.410	0.735	0.786	0.478	0.280
Idh-2	*a*	0.000	0.087	0.024	0.047	0.082	0.053	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000
	b	1.000	0.913	0.976	0.953	0.918	0.947	1.000	1.000	0.989	1.000	1.000	1.000	1.000	1.000