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GENETIC VARIATION IN NATURAL AND CULTIVATED POPULATIONS OF *PRIMULA VERIS*

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Primula veris, a well-known pharmaceutical plant, is a long-lived perennial protected by law in Poland, so its rhizomes and roots can be collected as pharmaceutical stock only from cultivation. Genetic variation of three natural *P. veris* populations numbering 500–1200 individuals and of three cultivated populations derived from seeds collected from natural sites was investigated in respect of two enzyme systems: phosphogluconate dehydrogenase (6PGD) and diaphorase (DIA). Four presumptive loci were identified from these two enzyme systems. In 6PGD, only one (6PGD-2) of two detected loci was polymorphic, consisting of three alleles *a, b* and *c*. Each of two electrophoretically detected loci in DIA was polymorphic and had two alleles. Comparison of the cultivated and natural populations revealed slight differentiation in the presence and composition of genotypes for 6PGD-2, while for DIA all populations except one preserved the same set of genotypes. Mean values of the polymorphism index for three loci ranged from 0.239 to 0.345 for natural populations and from 0.303 to 0.446 for cultivated populations, indicating that cultivated populations were more polymorphic than natural ones. The level of heterozygosity in the examined populations was very low. Mean values for Ho calculated for three polymorphic loci ranged from 0.033 to 0.056. The observed low heterozygosity level was confirmed by high values of Wright's fixation index, ranging from 0.798 to 0.910.

Key words: *Primula veris*, electrophoresis, genetic diversity, cultivation.

INTRODUCTION

The cowslip (*Primula veris* L.), of the Primulaceae family, a long-lived perennial heterostylous herb and an obligate outcrosser (Wedderburn and Richards, 1990), is a well-known pharmaceutical plant. Its rhizomes and roots, which are a pharmaceutical raw material (*Radix Primulae*), are composed of highly concentrated triterpene saponins (5–10%), mainly prymulasaponine A, prymulasaponine B, and phenolic glycosides such as prymulaverozyd and prymverozyd (Hegnauer, 1969, 1990; Calis et al. 1992; Kohlmünzer, 1993). Extracts from rhizomes, roots and flowers of *Primula veris* are components of such pharmaceutical specifics as Bronchicum, Pectosol, Grindmel-N, Tussipect and Sinupret (Neubauer and März, 1994; Strzelecka and Kowalski, 2000). Until now, *P. veris* has been collected in wild habitats of SE Europe, but this manner of plant collection endangers the survival of this species. In Poland *P. veris* is protected by law, so its cultivation should be combined with controlled collection from wild habitats. Valuable secondary metabolites produced by cowslip plants can be obtained only through cultivation of this plant. Seeds collected in natural populations from plants with a high level of biologically active substances may be used as a source of cultivated populations.

Primula veris is a species characterized by a very low level of genetic differentiation in natural

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populations (Antrobus and Lack, 1993; Morozowska and Krzakowa, 2003). This is surprising, as *P. veris* is distylous and strictly self-incompatible: only cross-pollination between two genetically determined pin and thrum flower morphs results in seed setting. Until now it was thought that one of the reasons for such a low level of genetic variation was the fact that reproduction of cowslip plants was realized mainly through vegetative propagation (Tamm, 1972; Morozowska, 1999, 2000). Recently, Kéry et al. (2000) reanalyzed data obtained by Tamm (1972) and found that 53% of new recruits were derived from seeds. The authors also cited a study of a small expanding population of *P. veris*, where 61% of recruitment was from the seeds and only 39% from clonal growth.

This study examines genetic variation between three natural cowslip populations, to find out whether cultivated populations derived from seeds collected from natural sites have the same or a different level of genetic differentiation.

MATERIALS AND METHODS

STUDY AREA

The study area was located in the Wielkopolska Lowland, W Poland, where three natural populations of *P. veris* were growing over 50 km apart under different environmental conditions. In populations 1, 2 and 3, cowslip plants were a component of a *Trifolio-Agrimonietum* association on light mineral soil (pH 4.7), a *Dactylis glomerata-Calamagrostis epigejos* community on humus-mineral neutral soil (pH 7.0), and a *Pruno-Crataegetum* association on mineral neutral soil (pH 7.1), respectively. The number of cowslip individuals was over 1200 in population 1, over 500 in population 2 and over 1000 in population 3. The mean number of individuals per square meter was 27.6, 8.1 and 28.6, respectively (Morozowska, 2000). The proportion of long- and short-styled flower morphs did not depart from a 1:1 ratio in population 1. In population 2, pin and thrum plants constituted 62.2% and 37.8%, whereas in population 3 there were 3.3% more plants with thrum flowers (Morozowska and Urbański, 2000). Population 1 was growing on the north edge of Wierzbiczańskie Lake in a protected zone, population 2 was within the Lednicki Landscape Park, and population 3 was within the Warta River Landscape Park.

Also studied were cultivated *P. veris* populations derived from seeds collected from the populations described above.

PLANT MATERIAL

Seeds from each natural population were collected randomly, and 1200 seeds from each population were sown in boxes and exposed to natural vernalization for three months (December–February). In March the seeds were taken into the greenhouse, where they germinated. The percentages of germinated seeds per population were 30.8% (pop. 1), 57.5% (pop. 2) and 26.5% (pop . 3). From the obtained seedlings about 300 per population were planted outside in the autumn of the same year. The plants were watered as needed. Almost all seedlings survived in the first year of cultivation; in the second year 2.5% of the plants died due to fungal infection. After three years, due to rhizome branching, the plants grew into several-rosetted individuals. The cultivated populations were labelled 1C, 2C and 3C.

Thirty randomly chosen individuals were sampled from each natural and cultivated population in the same year. Young leaves were collected from randomly selected individual plants in early spring, before flowering. In natural populations, selection was done along transects (sampled 1 m apart). Leaf samples were stored at 5˚C until the next day and then submitted to electrophoresis.

ELECTROPHORESIS PROCEDURE

Leaf tissue was homogenized with 0.1M Tris/HCL, pH 7.5, with 4% (w/v) polyvinylpyrrolidone (PVP). Electrophoresis was conducted horizontally in 11% starch gel under 60 mA at 4˚C for 5 h. All populations were examined in respect of two polymorphic enzyme systems: 6-phosphogluconate dehydrogenase (6PGD) and diaphorase (DIA). This was done because four others – menadione reductase (MNR), formate dehydrogenase (FDH), isocitrate dehydrogenase (IDH) and glutamate oxaloacetate transaminase (GOT) – proved to be monomorphic.

DATA ANALYSIS

Genetic parameters – observed heterozygosity (Ho), expected heterozygosity (He) and total genetic diversity (HT), mean diversity within populations (HS), fixation indices (F), polymorphism indices of genotypes (Pg), relative measure of genetic differentiation between populations (GST, DST) and genetic similarities between populations based on gene frequency (Nei, 1972), genotype frequencies (Hedrick, 1974) and genetic distances (DN, DH) – were calculated according to Jain and Workman (1967), Nei

(1978) and Kahler et al. (1980) using the GEN package (Nowak-Bzowy and Bzowy, unpubl.).

RESULTS

Four presumptive loci were identified from the two examined enzyme systems. Three of them were polymorphic in at least one population: 6PGD-2, DIA-1 and DIA-2. The average number of alleles per locus in a population ranged from 1.67 to 2.33. In 6PGD the polymorphic locus 6PGD-2 consisted of three alleles: *a*, *b* and *c*. Alleles *b* and *c* were present in all examined populations. In all populations allele *c* was the most frequent. The differences in its frequency were statistically significant between natural population 1 and cultivated population 2C and between population 3 and 2C. For allele *b*, statistically significant differences in its frequency were found between natural population 1 and cultivated populations 1C and 2C, and between population 3 and cultivated populations 1C and 2C. For DIA, each of the two electrophoretically detected loci had two alleles. All natural and cultivated populations were polymorphic according to the DIA-1 locus. Differences in the frequency of alleles *a* and *b* were statistically significant between populations 1 and 1C. According to DIA-2 locus, only one cultivated population (1C) was polymorphic (Tab. 1).

In all examined populations, five genotypes were observed for 6PGD-2 and three genotypes for DIA-1 and DIA-2. The mean number of genotypes per locus in each population ranged from 2.00 to 3.00, and the mean number of genotypes per locus for all examined populations was 2.397. For 6PGD-2, comparison of cultivated populations with natural ones revealed only slight differentiation in the presence and type of genotypes. Genotype *aa*, not found in natural populations 1 and 3, occurred infrequently in cultivated populations 1C and 3C; *bc* heterozygotes, infrequent in natural population 1, did not appear in cultivated population 1C. For DIA-1 and DIA-2, all populations preserved the same set of genotypes except population 1C, which for DIA-2 contained all the genotypes (Tab. 2). The mean values of the polymorphism index calculated for three loci ranged from 0.239 to 0.446 and were higher for cultivated populations. The highest Pg value was observed in population 1C. The mean value of Pg for all populations and three loci was 0.326 (Tab. 3). The Pg values for 6PGD confirmed that the cultivated populations were more polymorphic than natural ones, and the most polymorphic was population 2C. For DIA-1 the highest Pg values were found for natural populations 2 and 3, while cultivated populations 2C and 3C had slightly lower Pg values (Tab. 4).

In the six examined populations the mean values of observed heterozygosity (Ho) calculated for loci 6PGD-2, DIA-1 and DIA-2 were very low, rang-

TABLE 1. Allele frequencies of polymorphic loci in three natural and cultivated *Primula veris* populations

Locus	Allele	Population No.					
		1	2	3	1C	2C	3C
6PGD-2	*a*	0.000	0.100	0.000	0.033	0.089	0.022
	b	0.117	0.167	0.100	0.256	0.278	0.178
	c	0.883	0.733	0.900	0.711	0.633	0.800
DIA-1	*a*	0.300	0.417	0.400	0.550	0.389	0.389
	b	0.700	0.583	0.600	0.450	0.611	0.611
DIA-2	*a*	0.000	0.000	0.000	0.194	0.000	0.000
	b	1.000	1.000	1.000	0.806	1.000	1.000

TABLE 2. Genotype frequencies for three natural and cultivated *Primula veris* populations

Locus	Allele	Population No.					
		1	2	3	1C	2C	3C
6PGD-2	*aa*	0.000	0.100	0.000	0.033	0.089	0.022
	ac	0.000	0.000	0.000	0.000	0.000	0.000
	bb	0.100	0.167	0.100	0.256	0.278	0.178
	bc	0.033	0.000	0.000	0.000	0.000	0.000
	cc	0.867	0.733	0.900	0.711	0.633	0.800
DIA-1	*aa*	0.267	0.333	0.333	0.511	0.322	0.322
	ab	0.067	0.167	0.133	0.078	0.133	0.133
	bb	0.667	0.500	0.533	0.411	0.544	0.544
DIA-2	*aa*	0.000	0.000	0.000	0.178	0.000	0.000
	ab	0.000	0.000	0.000	0.033	0.000	0.000
	bb	0.100	0.100	0.100	0.789	0.100	0.100

TABLE 3. Genetic diversity estimates for the examined natural and cultivated *Primula veris* populations.
He – expected heterozygosity; Ho – observed heterozygosity; F – fixation index; Pg – polymorphism index

Population No.	He	Ho	F	Pg
1	0.209	0.033	0.840	0.239
2	0.304	0.056	0.817	0.345
3	0.220	0.044	0.798	0.257
1C	0.412	0.037	0.910	0.446
2C	0.330	0.044	0.865	0.365
3C	0.268	0.044	0.834	0.303
Mean	0.291	0.043	0.844	0.326

TABLE 4. Genetic diversity estimates per locus among the examined natural and cultivated *Primula veris* populations. He – expected heterozygosity; Ho – observed heterozygosity; F – fixation index; Pg – polymorphism index

Locus	Population No.	He	Ho	F	Pg
6PGD-2	1	0.206	0.033	0.838	0.238
	2	0.424	0.000	1.000	0.424
	3	0.180	0.000	1.000	0.180
	1C	0.428	0.000	1.000	0.428
	2C	0.514	0.000	1.000	0.514
	3C	0.328	0.000	1.000	0.328
DIA-1	1	0.420	0.067	0.841	0.480
	2	0.486	0.167	0.657	0.611
	3	0.480	0.133	0.722	0.587
	1C	0.495	0.078	0.843	0.563
	2C	0.475	0.133	0.715	0.582
	3C	0.475	0.133	0.719	0.582
DIA-2	1C	0.313	0.033	0.894	0.345

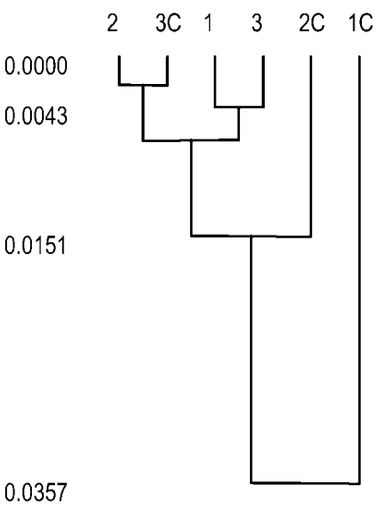

Fig. 1. Dendrogram illustrating genetic similarities of *Primula veris* populations based on allele frequencies.

TABLE 5. Genetic parameters estimating variation within and among the examined populations of *Primula veris*. HT – total genetic diversity; HS – mean diversity within populations; DST – and GST – measures of genetic differentiation between populations

Locus	HT	HS	DST	GST
6PGD-2	0.398	0.385	0.013	0.032
DIA-1	0.489	0.477	0.012	0.024
DIA-2	0.093	0.078	0.014	0.153
Mean	0.326	0.313	0.013	0.039

TABLE 6. Nei's (DN) and Hedrick's (DH) genetic distances between the examined *Primula veris* populations

Numbers of pair-wise compared populations		DN	DH
2	1	0.012	0.018
2	3	0.008	0.011
2	2C	0.006	0.007
2	1C	0.029	0.038
2	3C	0.003	0.003
1	3	0.004	0.006
1	2C	0.023	0.028
1	1C	0.052	0.064
1	3C	0.005	0.008
3	2C	0.023	0.027
3	1C	0.035	0.048
3	3C	0.003	0.004
2C	1C	0.035	0.040
2C	3C	0.009	0.011
1C	3C	0.030	0.039

ing from 0.033 to 0.056. The Ho mean value for all populations and three loci was 0.043, with a heterozygosity expected (He) value of 0.291 (Tab. 3). For 6PGD-2, in five of the six examined populations the Ho was equal to zero. For DIA-1 and DIA-2 the level of heterozygosity was also low and was confirmed by high values of Wright's fixation index (F = 0.657–0.894). The highest heterozygosity level was observed for natural populations 2 and 3, and for cultivated populations 2C and 3C (Tab. 4).

The mean value of the level of genetic differentiation within examined populations, HS = 0.313, was higher than between populations GST = 0.039 (total genetic diversity HT = 0.326). For 6PGD-2 the GST value was 0.032 and the highest genetic variation between the six examined populations was found for DIA-2 (15.3%) (Tab. 5).

The dendrogram (Fig. 1) shows the genetic similarities between the examined populations based on allele frequencies. The populations were compared with the agglomeration method and closest neighborhood technique (UPGMA). Nei's (1972) genetic distances for each pairwise comparison of populations ranged from 0.003 to 0.052, and according to Hedrick's (1974) method of evaluation the genetic distances ranged from 0.003 to 0.064. The populations formed one cluster consisting of four of them (2, 3C, 1 and 3). Population 1C and also population 2C were clearly different from them (Tab. 6).

DISCUSSION

The levels of genetic variation in plant populations are highly variable (Hamrick and Godt, 1990). In the present work the very low level of genetic differentiation found in natural cowslip populations confirms similar results reported by other authors (Antrobus and Lack, 1993; Morozowska and Krzakowa, 2003). The level of genetic variation among the examined populations was similar to that found in English populations, where the GST value ranged from 0.025 for 6PGD-1 to 0.050 for 6PGD-2 (Antrobus and Lack, 1993). Our results were much lower than the mean values for animal-outcrossed species and species with gravity-dispersed seeds reported by Hamrick and Godt (1990).

In England, where the genetics of colonizing and established populations of *P. veris* was examined (Antrobus and Lack, 1993), the authors expected that colonizing populations will show evidence of the founder effect, but they found that any possible reduction in gene diversity was slight and that differentiation between colonizing populations was not greater than that between established ones. They suggested that colonizing populations have been initiated by many individuals (Antrobus and Lack, 1993). This is in agreement with Sirkkomaa's (1983) theoretical explanation: the genetic structure of populations with rare alleles is affected mainly by the number of founders, even after many generations. Our results showed that cultivated populations, each of which was started from about 300 individuals, were slightly more polymorphic than natural ones. This might come about as a result of many individuals being involved in founding these populations.

Interesting and surprising was the very low level of observed heterozygosity found in the examined populations, especially in cultivated populations started from seeds of *P. veris*, an obligate outcrosser. Probably the different alleles of the examined genes coding such enzyme systems as 6-phosphogluconate dehydrogenase and diaphorase were not present in the examined populations from natural stands.

Comparison of genetic similarities based on allele frequencies between natural and cultivated populations showed that the lowest values of mean Nei's genetic distances were between populations 3 and 3C, and the most separate were populations 1 and 1C. The difference between populations 1 and 1C was also clear in allele and genotype frequencies, and in the statistically significant difference in allele frequencies between these two populations,

At least according to the examined enzyme systems, the cowslip plants in the cultivated populations had a slightly higher level of genetic variation than their initial population. When we carried out genetic experiments, we evaluated saponin content in rhizomes and roots of *P. veris* plants from the examined populations and found that cowslip plants in cultivated populations not only maintain the same level of saponin content as in the initial populations but also tend to increase it, in a range of 0.8% to 2.3% depending on the population (Morozowska, unpubl. data). This should encourage medicinal plant cultivators to expand *Primula veris* propagation with the aim of conserving natural stands.

REFERENCES

ANTROBUS S, and LACK AJ. 1993. Genetics of colonizing and established populations of *Primula veris*. *Heredity* 71: 252–258.

CALIS I, YÜRÜKER A, RÜEGGER H, WRIGHT AD, and STICHER O. 1992. Triterpene saponins from *Primula veris* subsp. *macrocalyx* and *Primula elatior* subsp. *meyeri*. *Journal of Natural Products* 55: 1299–1306.

HAMRICK JL, and GODT MJ. 1990. Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS [eds.], *Plant population genetics, breeding and genetic resources*, 43–63. Sinauer Associates Inc., MA.

HEDRICK PW. 1974. Genetic similarity and distance; comments and comparisons. *Evolution* 29: 362–366.

HEGNAUER R. 1969. *Chemotaxonomie der Pflanzen*. Band 5, 389–393. Birkhäuser Verlag, Basel, Stuttgart.

HEGNAUER R. 1990. *Chemotaxonomie der Pflanzen*. Band 9, 289–292. Birkhäuser Verlag, Basel, Boston, Berlin.

JAIN SK, and WORKMAN PW. 1967. Generalized F-statistics and the theory of inbreeding and selection. *Nature* 214: 674–678.

KAHLER AL, ALLARD RW, KRZAKOWA M, WEHRHAN CF, and NEVO E. 1980. Associations between isozyme phenotypes and environment in the slender wild oat (*Avena barbara*) in Israel. *Theoretical and Applied Genetics* 56: 31–47.

KÉRY M, METTHIES D, and SPILLMAN HH. 2000. Reduced fecundity and offspring performance in small populations of the declining grassland plants *Primula veris* and *Gentiana lutea*. *Journal of Ecology* 88: 17–30.

KOHLMÜNZER S. 1993. *Farmakognozja*. PZWL, Warszawa.

MOROZOWSKA M. 1999. Importance of studies on population biology for species protection of medicinal plants on an example of selected populations of cowslip (*Primula veris* L.). *Roczniki Akademii Rolniczej w Poznaniu, CCCX, Melior. Inż. Środ.* 20, pt. II: 437–445.

MOROZOWSKA M. 2000. Charakterystyka ekologiczna naturalnych populacji *Primula veris* L. na Nizinie Wielkopolskiej. *Roczniki Akademii Rolniczej w Poznaniu, CCCXXII, Botanika* 3: 165–173.

MOROZOWSKA M, and KRZAKOWA M. 2003. Genetic variation of cowslip (*Primula veris* L.) populations (West Poland). *Acta Societatis Botanicorum Poloniae* 72: 331–336.

MOROZOWSKA M, and URBAŃSKI Ł. 2000. Charakterystyka kwitnienia i owocowania oraz ocena produkcyjności nasiennej roślin pierwiosnka lekarskiego (*Primula veris* L.) z populacji zlokalizowanych na Nizinie Wielkopolskiej. *Roczniki Akademii Rolniczej w Poznaniu, CCCXXII, Botanika* 3: 175–184.

NEI M. 1972. Genetic distance between populations. *American Naturalist* 106: 283–292.

NEI M. 1978. *Molecular evolutionary genetics*. Columbia University Press, New York.

NEUBAUER N, and MÄRZ RW. 1994. Placebo-controlled, randomised double-blind clinical trial with Sinupret sugar coated tablets on the basis of a therapy with antibiotics and decongestant nasal drops in acute sinusitis. *Phytomedicine* 1: 177–181.

SIRKKOMAA S. 1983. Calculations on the decrease of genetic variation due to the founder effect. *Hereditas* 99: 11–20.

STRZELECKA H, and KOWALSKI J. 2000. *Encyklopedia zielarstwa i ziołolecznictwa*. PWN, Warszawa.

TAMM CO. 1972. Survival and flowering of perennial herbs III. The behaviour of *Primula veris* on permanent plots. *Oikos* 23: 159–166.

WEDDERBURN F, and RICHARDS AJ. 1990. Variation in within-morph incompatibility sites in heteromorphic *Primula* L. *New Phytologist* 116: 149–162.