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GENETIC DIFFERENTIATION AMONG GEOGRAPHICALLY CLOSE POPULATIONS OF *MALVA ALCEA*

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We estimated similarity and differences at the level of isozymes among populations of *Malva alcea* from various habitats and parts of Central Europe. Our analyses revealed the activity of 8 enzyme systems: 6 polymorphic (PGI, IDH, PX, DIA, PGM, SHD KB) and 2 monomorphic (GOT, ME). The mean number of alleles per locus is 2.12. Nei's genetic distances among populations are small, not exceeding 0.3. Mean values of Nei's genetic similarity are typical of populations of the same species. Grouping by UPGMA based on Nei's genetic distances showed that the distinguished groups are only partly correlated with geographic region.

Key words: *Malva alcea*, isozymes, genetic differentiation, Central Europe, Poland.

INTRODUCTION

Species of 10 genera of the family Malvaceae are found in Central Europe, but some of them are only cultivated there. The genus *Malva* is the richest in species, as it includes from 6 to 11 species depending on the classification (e.g., Mirek et al., 2002; Slavík, 2002; Rothmaler et al., 2005). The most common among them is *M. alcea*. This species was used from prehistoric times through the Middle Ages for various purposes: as a medicinal and food plant, for dyeing, and for ornament or magic. It now is only rarely cultivated, as an ornamental plant, and is considered a relic of cultivation (Celka and Drapikowska, 2008). According to some authors, in eastern Poland *M. alcea* is at the limit of its continuous range of distribution (Walas, 1959; Il'in, 1974). Further east a closely related species, *M. excisa*, is found (Dobrochaéva, 1955; Il`in, 1974; Zubkevich, 1999), and *M. alcea* is encountered only in the Baltic states (Kuusk et al., 1996; Koltzenburg, 2001). *M. excisa* is sometimes classified within *M. alcea* as a subspecies or variety (Hegi, 1925; Rutkowski, 2004).

The taxonomic position and morphological variation of *M. alcea* has been the subject of numerous publications. Previous studies examined variation of petal shape in selected populations of *M. alcea* from Central and Eastern Europe (Celka et al., 2007), seeds and seed structures (Kumar and Singh, 1991; Celka et al., 2006b) and hairs (Inamdar and Chohan, 1969; Inamdar et al., 1983; Celka et al., 2006a). Also studied was the persistence and spread of *M. alcea* in and near archeological sites (Celka et al., 2008) and the evolution of species in the Malva alliance, based on molecular markers (Escobar García et al., 2009). So far there has been no analysis of the variation of isozyme markers in *M. alcea* populations.

With the use of electrophoretic separation of proteins, the degree of polymorphism can be estimated in individual populations and differences among populations can be analyzed (Soltis and Soltis, 1989; Crawford, 1989). This method also makes it possible to investigate the genetic structure of a species (Gottlieb, 1981). Isozyme data can provide information about the biology of studied species, for example the mode of reproduction, clonal structure, mating system and gene flow among populations (Hamrick and Godt 1989; Brown and Schoen 1992; Diggle et al., 1998; Guo et al., 2003). Isozyme studies have analyzed the genetic structure

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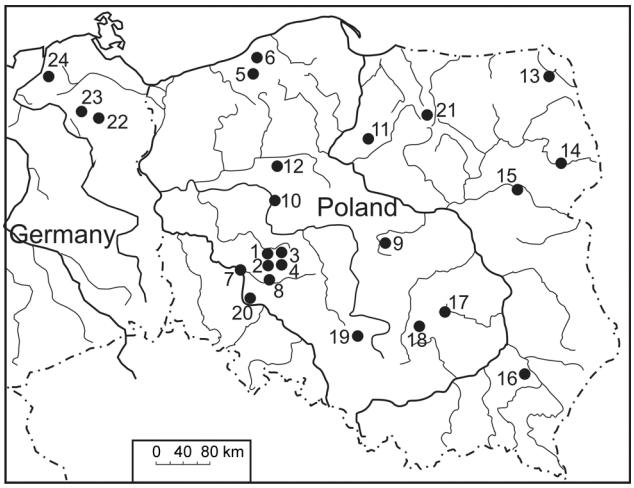

Fig. 1. Geographic location of studied populations (population numbers – see Appendix 1)

of rare and endangered species such as *Cypripedium calceolus* (Brzosko et al., 2002a; Brzosko et al., 2002b), the correlation between genetic diversity and geographic distance in *Piper cernuum* (Mariot et al., 2002) and *Viola odorata* (Marcussen, 2006), the association of genetic variation with environmental differences (Diggle et al., 1998; Guo et al., 2003) and gene flow among closely related taxa of cultivated and free-living *Cucurbita* (Montes-Hernandez and Eguiarte, 2002). Isozymes have also been used to resolve taxonomical problems (e.g., López-Pujol et al., 2006; Caliskan et al., 2006; Frizzi et al., 2007).

There are no published reports on isozyme variation in *M. alcea*. In this study we estimate similarity and differences at the level of isozymes among populations of *M. alcea* from different habitats in various parts of Central Europe. The aim is to throw new light on taxonomic problems in the *M. alcea* complex.

MATERIALS AND METHODS

SPECIES STUDIED

Malva alcea is distributed in Central, Southern and part of Western Europe, as well as southern Sweden and Denmark. Isolated populations are found in Spain, Sardinia and Corsica, along the Black Sea and in North America (Hultén and Fries, 1986). It is a perennial, autogamous and entomophilous plant, reproducing by seeds and vegetatively by division of the main root (Łukasiewicz, 1962; Klotz et al., 2002; Moraczewski et al., 2008). *Malva alcea* grows on warm, sunny sites on calcareous and nitrogen-rich soils. Its major sites are associated with places where it was cultivated in the Middle Ages: hill forts, castles, and other former human settlements. As a result of spreading from sites of cultivation it is now found very often in other anthropogenic habitats as well: roadsides, ditches along roads, edges of shrub communities, and ruderal sites (Hegi, 1925; Bauch, 1937; Walas, 1959; Rothmaler et al., 2005; Celka and Drapikowska, 2008). It is associated with nitrophilous communities of perennials on ruderal sites (Matuszkiewicz, 2001; Zarzycki et al., 2002; Rothmaler et al., 2005).

FIELD STUDY

For isozyme analyses, plant material was collected from 24 populations of *M. alcea* in summer in 2006 and 2007 (Fig. 1). The populations were grouped into three regions delimited by two big rivers, the Oder and Vistula: I – western, including 3 sites from Mecklenburg in Germany; II – central, including 15 populations from western and central Poland between the Oder and the Vistula; and III – eastern, including 6 populations in eastern Poland (Appendix 1). The collected samples represent the major habitats of *M. alcea*: medieval fortified settlements, roadsides, roadside ditches, edges of forest and shrub communities, flood embankments, and a cemetery (see Appendix 1). For statistical analyses, habitats were grouped in 3 main types: grassland, roadside and thicket (see Table 2). Between 2 and 43 individuals from each population were studied (total 1232). At 6 of the 24 localities (sites 2, 6, 8, 14, 17, 22) samples were collected from only 2–5 individuals; these populations were included in the analysis because they are geographically isolated from the others. At these six localities, samples were collected from all individuals of *M. alcea*. Each sample was divided into two parts, one of which was kept at 4°C until isoenzymatic analysis; the other was deposited in the Herbarium of the Department of Plant Taxonomy, Adam Mickiewicz University, Poznań, Poland (POZ).

ELECTROPHORESIS

We studied the variation of 8 enzyme systems: PGI – phosphoglucoisomerase (EC.5.3.1.9.); PX – peroxidase (EC 1.11.1.7); GOT – glutamate oxaloacetic transaminase (EC 2.6.1.1); DIA – NADH diaphorase (EC 1.6.2.2); IDH – isocitrate dehydrogenase (EC 1.1.1.42); ME – malic enzyme (EC 1.1.1.40); PGM – phosphoglucomutase (EC 5.4.2.2); and SHD – shikimate dehydrogenase (EC 1.1.1.25). Isozymes were separated on 10% starch gel in two buffer systems: PGI, PX, GOT, and DIA in tris-citrate (pH 8.2)/lithium-borate (pH 8.3); and IDH, ME, PGM, and SHD in morpholine-citrate (pH 6.1), in a 1:14 dilution of electrode buffer (Wendel and Wenden, 1989). Lithium-borate gels were separated at constant volt-

TABLE 1. Allele frequencies at polymorphic enzymatic loci in 24 populations of *Malva alcea*

Locus	Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Pgi-1	A	0.546	1.000	0.480	0.650	0.500	0.500	0.473	0.875	0.800	0.780	0.764	0.750	0.803	0.333	0.750	0.667	0.667	0.771	0.852	0.774	0.833	0.500	0.645	0.828
	B	0.454	–	0.520	0.350	0.500	0.500	0.527	0.125	0.200	0.220	0.236	0.250	0.197	0.667	0.250	0.333	0.333	0.229	0.148	0.226	0.167	0.500	0.355	0.172
Pgi-2	A	0.639	0.500	0.680	0.550	0.807	0.900	0.662	0.500	0.750	0.720	0.667	0.488	0.712	1.000	0.583	0.867	0.833	0.771	0.833	0.758	0.650	0.750	0.887	0.906
	B	0.361	0.500	0.320	0.450	0.193	0.100	0.338	0.500	0.250	0.280	0.333	0.512	0.288	–	0.417	0.133	0.167	0.229	0.167	0.242	0.350	0.250	0.113	0.094
Idh	A	0.631	0.750	0.740	0.350	0.455	0.700	0.392	0.250	0.500	0.540	0.556	0.650	0.697	0.500	0.458	0.617	0.500	0.671	0.778	0.742	0.817	0.875	0.645	0.641
	B	0.369	0.250	0.260	0.650	0.545	0.300	0.608	0.750	0.500	0.460	0.444	0.350	0.303	0.500	0.542	0.383	0.500	0.329	0.222	0.258	0.183	0.125	0.355	0.359
Px-1	A	0.215	–	0.180	–	0.034	–	0.027	–	–	0.100	0.014	0.050	0.091	–	0.021	–	–	–	0.148	0.242	0.117	–	0.323	0.266
	B	0.177	0.250	0.400	–	0.125	0.300	0.095	0.500	0.238	0.280	0.431	0.325	0.242	0.167	0.021	0.033	0.167	0.014	0.241	0.129	0.217	0.375	0.226	0.281
	C	0.446	0.250	0.320	0.600	0.648	0.500	0.703	0.250	0.525	0.460	0.347	0.175	0.561	0.833	0.875	0.733	0.666	0.700	0.426	0.355	0.566	0.500	0.403	0.344
	D	0.162	0.500	0.100	0.400	0.193	0.200	0.175	0.250	0.237	0.160	0.208	0.450	0.106	–	0.083	0.234	0.167	0.286	0.185	0.274	0.100	0.125	0.048	0.109
Px-2	A	0.331	–	0.620	0.750	0.409	0.300	0.500	0.375	0.613	0.420	0.583	0.687	0.697	0.500	0.792	0.800	1.000	0.971	0.111	0.226	0.217	0.125	0.081	0.125
	B	0.669	1.000	0.380	0.250	0.591	0.700	0.500	0.625	0.387	0.580	0.417	0.313	0.303	0.500	0.208	0.200	–	0.029	0.889	0.774	0.783	0.875	0.919	0.875
Px-3	A	0.708	1.000	0.780	0.750	0.500	0.500	0.595	1.000	0.725	0.660	0.625	0.538	0.561	0.500	0.938	0.850	0.833	0.829	0.833	0.806	0.917	0.750	0.274	0.297
	B	0.292	–	0.220	0.250	0.500	0.500	0.405	–	0.275	0.340	0.375	0.462	0.439	0.500	0.062	0.150	0.167	0.171	0.167	0.194	0.083	0.250	0.726	0.703
Dia	A	0.746	1.000	0.660	0.500	0.693	0.600	0.662	0.750	0.787	0.760	0.639	0.687	0.894	0.500	0.937	0.850	1.000	0.829	0.889	0.758	0.417	0.750	0.742	0.813
	B	0.254	–	0.340	0.500	0.307	0.400	0.338	0.250	0.213	0.240	0.361	0.313	0.106	0.500	0.063	0.150	–	0.171	0.111	0.242	0.583	0.250	0.258	0.187
Sdh	A	0.277	0.500	0.240	0.250	0.114	0.400	0.297	0.250	0.325	0.100	0.361	0.375	0.773	0.500	0.813	0.767	0.833	0.871	0.593	0.871	0.917	0.750	0.855	0.844
	B	0.723	0.500	0.760	0.750	0.886	0.600	0.703	0.750	0.675	0.900	0.639	0.625	0.227	0.500	0.187	0.233	0.167	0.129	0.407	0.129	0.083	0.250	0.145	0.156
Pgm	A	0.777	0.500	0.720	0.900	0.716	0.500	0.851	0.875	0.825	0.720	0.875	0.900	0.742	1.000	0.875	0.867	0.833	0.886	0.796	0.855	0.650	0.625	0.823	0.844
	B	0.223	0.500	0.280	0.100	0.284	0.500	0.149	0.125	0.175	0.280	0.125	0.100	0.258	–	0.125	0.133	0.167	0.114	0.204	0.145	0.350	0.375	0.177	0.156

age, and morpholine-citrate gels were separated at constant current. Crude cell extract was prepared by homogenizing a small piece of young leaf in 80 µl extraction buffer (Gottlieb, 1981). After separation, the enzymes were stained by standard methods (Wendel and Wenden, 1989).

DATA ANALYSIS

Allele frequencies, percentage of polymorphic loci (P), mean number of alleles per locus (A), and observed and expected heterozygosity (H_O, H_E) were estimated for each population. Means of these statistics for different habitat types were calculated and the Kruskal-Wallis ANOVA test was used to check their statistical significance. To detect the possible effects of selection the Ewens-Watterson test for neutrality (1000 permutations) (Manly, 1985) was performed for each locus. Linkage disequilibrium among all pairs of loci was also tested. The mean fixation index (F_{IS}) Wright (1978) for all polymorphic loci in each population as well as over all populations for each locus was used as a relative measure of deviations of populations from Hardy-Weinberg equilibrium (HWE), that is, increased or decreased heterozygosity as compared with the level expected for random mating. To test for HWE we used the chi-square (χ^2) test. We also calculated gene flow (N_m) from the formula $N_m = 0.25 \times (1 - F_{ST})/F_{ST}$, where F_{ST} is the coefficient of genetic differentiation (Wright, 1951). Unbiased genetic distance (D) and genetic identity (I) between pairs of populations were computed according to Nei (1978). We constructed a UPGMA dendrogram and performed principal coordinate analysis (PCA) based on the matrix of Nei's (1978) genetic distance. The analyses employed POPGENE-1.32. (Yeh and Boule, 2000), GenAlEx 6.3 (Peakall and Smouse, 2006) and STATISTICA 7.1 for Windows (StatSoft 2008).

To investigate the genetic structure of populations we performed hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992). First we used AMOVA to estimate the partitioning of the total genetic diversity among and within the studied populations. Then we calculated it with regard to the division of the studied populations into three regions of populations, and among populations within each group as well as within populations. The level of genetic differentiation was estimated using F statistics (F_{IS}, F_{IT}, F_{ST}) calculated within the AMOVA framework. A permutation test (1000 permutations) was used to check the significance of F statistics, and a Mantel line regression test (Mantel, 1976) to test for the correlation between genetic distance and geographic distance among populations. The number of permutations in the Mantel test was 1000. The above were performed with GenAlEx 6.3 (Peakall and Smouse, 2006).

RESULTS

The analyses revealed the existence of 11 loci, of which 9 were polymorphic (*Pgi*-1, *Pgi*-2, *Idh*, *Px*-1, *Px*-2, *Px*-3, *Dia*, *Shd*, *Pgm*), and the other 3 monomorphic (*Got, Me* and *Idh-2*). For each locus

TABLE 2. Genetic variation of the studied populations of *Malva alcea*

No. of populations	Sample size	A	P	H_O	H_E	F_{IS}
grassland						
1	65	2.1	90	0.463	0.414	-0.118
2	2	1.6	50	0.400	0.333	-0.201*
3	25	2.1	90	0.504	0.413	-0.220*
9	40	2.0	90	0.438	0.379	-0.156
11	36	2.1	90	0.419	0.410	-0.022
12	40	2.1	90	0.485	0.404	-0.200
Mean of grassland	34.7	2.0	83.3	0.452	0.392	-0.153
roadside						
4	10	1.9	90	0.440	0.388	-0.134
6	5	2.0	90	0.580	0.456	-0.272
7	37	2.1	90	0.465	0.404	-0.151
8	4	1.9	80	0.275	0.361	0.238*
10	25	2.1	90	0.404	0.388	-0.041
13	33	2.1	90	0.352	0.365	0.036
14	3	1.7	70	0.467	0.378	-0.235*
17	3	1.8	70	0.333	0.307	-0.085
18	35	2.0	90	0.280	0.266	-0.053
19	27	2.1	90	0.330	0.312	-0.058
20	31	2.1	90	0.403	0.338	-0.192
21	30	2.1	90	0.300	0.328	0.085
22	4	2.0	90	0.500	0.400	-0.250*
23	31	2.1	90	0.323	0.332	0.027
Mean of roadside	19.9	2.0	86.4	0.389	0.359	-0.078
thicket						
5	44	2.1	90	0.461	0.390	-0.182
16	30	2.0	90	0.360	0.302	-0.192
24	32	2.1	90	0.350	0.315	-0.111
15	24	2.1	90	0.288	0.273	-0.055
Mean of thicket	32.5	2.1	90.0	0.365	0.320	-0.135
Overall mean		2.01	86.3	0.399	0.361	-0.101

Explanations: A – mean number of alleles per locus; P – percentage of polymorphic loci; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – fixation index; * – $p \leq 0.05$

the number of alleles and genotypes was determined and their frequencies were calculated (Table 1). The statistical significance of differences in frequency of alleles and genotypes was confirmed with the chi-square test.

In the PGI system, two loci were detected and denoted: *Pgi*-1 (alleles A and B, with frequencies 0.691 and 0.309 respectively) and *Pgi*-2 (alleles A and B, with frequencies 0.721 and 0.279). In the IDH system two loci were detected: *Idh*-1 (alleles A and B, with frequencies 0.608 and 0.392) and *Idh*-2 which was monomorphic. In the PX system three loci were found: *Px*-1 (alleles A, B, C, D, with frequencies 0.102, 0.203, 0.502, 0.193), *Px*-2 (alleles A and B, with frequencies 0.479 and 0.520) and *Px*-3 (alleles A and B, with frequencies 0.666 and 0.334). Single loci were detected in the DIA system (alleles A and B, with frequencies 0.742 and 0.258), in the SHD system (alleles A and B, with frequencies 0.510 and 0.480), and in the PGM system (alleles A and B, with frequencies 0.806 and 0.194).

A comparison of allele frequencies in individual populations (Tab. 1) shows that in *Pgi*-1 and *Pgi*-2 the first alleles prevail. An analysis of frequencies of Idh alleles indicates that in most populations the allele *Idh*-A is most frequent, except in populations 9, 14 and 17, where both alleles are equally frequent, and populations 4, 5, 7, 8 and 15, where allele *Idh*-B is more frequent. In *Px*-1, allele A is the rarest, as it is absent from ten populations, while in the other populations it is less frequent than other alleles. In *Px*-3, allele A dominates in populations 1–4, 7–13 and 15–17, alleles A and B are equally frequent in populations 5, 6 and 14, while allele B prevails in populations 19–24. In *Dia, Shd* and *Pgm* the first alleles prevail in most populations. Among the

TABLE 3. Wright's (1965) F statistics for all studied populations at 9 polymorphic loci

Locus	F_{IS}	F_{IT}	F_{ST}	N_m
Pgi-1	-0.219	-0.131	0.073***	3.195
Pgi-2	-0.188	-0.125	0.053***	4.509
Idh-1	-0.038	0.013	0.049***	4.509
Px-1	-0.005	0.079**	0.084***	2.738
Px-2	-0.120	0.167**	0.257***	0.724
Px-3	-0.047	0.099*	0.139***	1.548
Dia	-0.141	-0.072	0.060***	3.898
Sdh	-0.249	0.161	0.328***	0.511
Pgm	-0.024	0.000	0.023***	10.430
Mean	-0.107	0.033*	0.126***	1.734

Explanations: * – p≤0.05; ** – p≤0.01; *** – p≤0.001

TABLE 4. Ewens-Watterson test for neutrality for all studied populations

Locus	n	k	Obs. F	L96*	U95*
Pgi-1	1232	2	0.5728	0.5078	0.9984
Pgi-2	1232	2	0.5975	0.5061	0.9984
Idh-1	1232	2	0.5233	0.5046	0.9984
Idh-2	1232	1	1.0000	-	-
Px-1	1232	4	0.3406	0.3447	0.9871
Px-2	1232	2	0.5008	0.5030	0.9984
Px-3	1232	2	0.5554	0.5027	0.9984
Dia	1232	2	0.6170	0.5037	0.9984
Sdh	1232	2	0.5007	0.5022	0.9984
Pgm	1232	2	0.6873	0.5026	0.9984

analyzed populations, population 2 includes as many as four monomorphic loci (*Pgi*-1, *Px*-2, *Px*-3, *Dia*) but this results from the small size of this population. It should be noted that we did not find any private alleles (i.e., alleles unique to one population).

In individual populations the mean number of alleles per locus ranged from 1.6 to 2.1 in grassland, from 1.7 to 2.1 on roadsides, and from 2.0 to 2.1 in thicket (Table 2). Grassland populations had the highest H_O, H_E and F values and the lowest P value. The Kruskal-Wallis ANOVA test showed no statistically significant differences among three of the studied habitat types in respect of all statistics. Combined analysis of all populations showed that most populations were highly polymorphic (90%); the percentage of polymorphic loci was lower in populations 2 (50%), 14 and 17 (70% each) and 8 (80%). Values for observed heterozygosity (H_O) were high in all populations, ranging from 0.275 in population 8 to 0.580 in population 6. In eight populations (1, 3, 5, 7, 9, 12, 14, 22) H_O was ~0.5. In nearly all populations, H_O was higher than H_E (which represents the frequency of heterozygotes expected for a population in H-W equilibrium). Populations 10, 11, 13, 15, 17–19, 21 and 23 are very close to H-W equilibrium ($F_{IS} \approx 0$). In many populations an excessive proportion of heterozygotes was found ($F_{IS} < 0$), indicating that allogamy prevails (Tab. 2). Deviation from H-W equilibrium was statistically significant in five small populations: 2, 3, 8, 14 and 22. The mean fixation index (F_{IS}) for all populations and for each locus had statistically nonsignificant negative values (Tab. 3).

The Ewens-Watterson test for neutrality for each locus (Tab. 4) showed that the allele frequencies at all loci were selectively neutral in the studied populations. A test for linkage disequilibrium among loci demonstrated the presence of linkage in only 16 populations, between loci PX2 and PX3. The

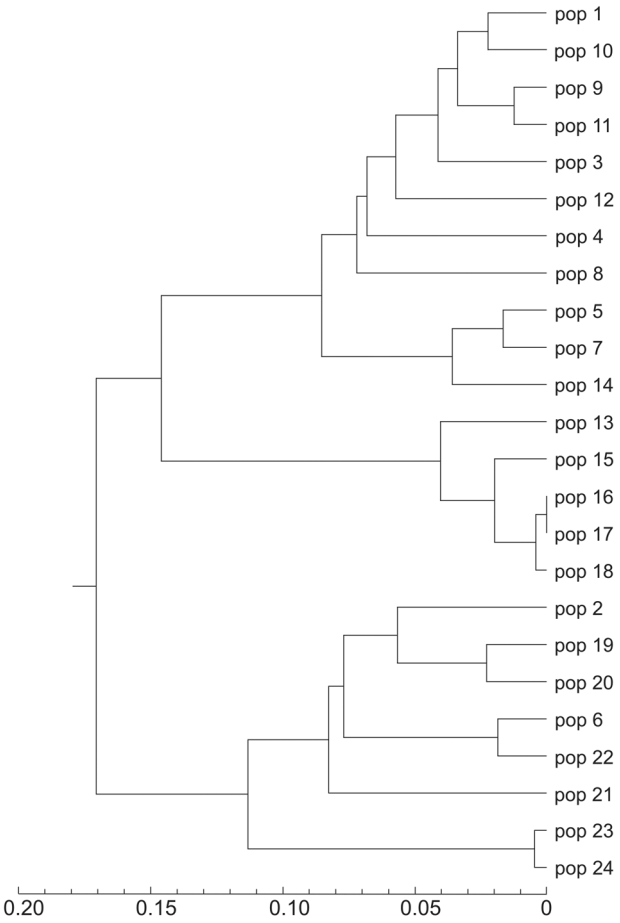

Fig. 2. UPGMA dendrogram of 24 populations of *Malva alcea* based on Nei's (1978) genetic distance (population numbers – see Appendix 1)

data were checked for the presence of clones; the proportion of clones was small, with only 35 repeated genotypes of the 616 studied (5.7%). For this reason the repeated genotypes were not excluded and

TABLE 5. Hierarchical analysis of molecular variance (AMOVA) without (A) and with (B) division of the studied populations into three regions

Source of variation	*df*	Sum of squares	Variance component	Variance %	Fixation index
A					
Among populations	23	339.78	0.261	12	F_{ST} =0.126***
Within populations	592	1232.50	2.001	88	
B					
Among regions	2	96.57	0.106	5	F_{RT}=0.050***
Among populations within regions	21	243.21	0.202	9	F_{SR}=0.101***
Within populations	592	1232.50	2.001	87	F_{ST}=0.146***

Explanations: A: F_{ST} – variation among populations divided by total variation; B: F_{RT} – variation among regions divided by total variation; F_{SR} – variation among populations within regions divided by the sum of variation among populations within regions and variation within populations; F_{ST} – sum of variation among regions and variation among populations divided by total variation; *** – P≤0.001

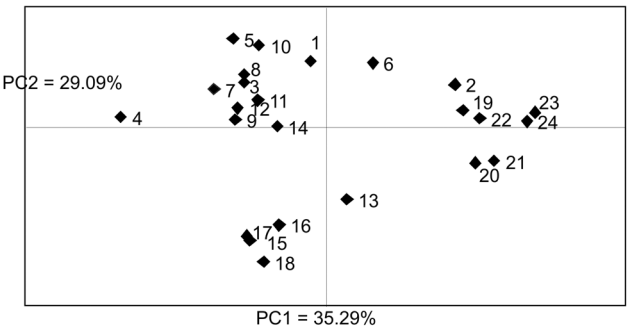

Fig. 3. Scatter plot of PCA based on Nei's (1978) genetic distance among 24 populations of *Malva alcea*. Percentage of explained variability: PC1 = 35.29%. PC2 = 29.09%

calculations were made for the whole data matrix.

AMOVA for the studied populations revealed that most of the genetic variation (88%) was within populations (Table 5). AMOVA with division into three regions permitted partitioning of most of the variation within population (87%). The remaining genetic variation was distributed among populations within regions (9%). Genetic variation was smallest among regions (5%) but was statistically significant ($p < 0.001$) (Tab. 5). To estimate genetic similarity among the studied populations we calculated Nei's (1978) genetic distances (Fig. 2). Two large groups of populations emerged. The first diverged into two subgroups: subgroup 1 (populations 1, 10, 9, 11, 3, 12, 4, 8, 5, 7, 14) and subgroup 2 (populations 13, 15, 16, 17, 18). The second group comprised populations 2, 19, 20, 6, 22, 21, 23 and 24. No correlation between location on the dendrogram and habitat type or the geographic origin of individual populations was found.

Principal coordinate analysis based on Nei's (1978) genetic distances grouped the populations into three groups. Group 1 includes populations 1, 3, 5, 7, 8, 9, 10, 11, 12 and 14; group 2 consists of populations 2, 19, 20, 21, 22, 23 and 24; and group 3 comprises populations 13, 15, 16, 17 and 18. Population 4 clearly diverges from all these groups, while population 6 is situated between groups 1 and 2. The PCA diagram in Figure 3 is consistent with the dendrogram (Fig. 2). The Mantel test showed no significant correlation between the genetic distance matrix and geographic distance matrix (0.32, $p > 0.05$).

DISCUSSION

This study of isozyme variation in *M. alcea* is the first report on this subject. Our results from Central Europe show relatively low genetic differentiation of this species. Nearly all the studied isoenzymatic loci were polymorphic (except *Me, Got* and *Idh*-2), but the numbers of alleles at individual loci were low (2.01). Interpopulation variation was also low (F_{ST}=0.126). The literature gives some information about high morphological variation in *M. alcea* (Walas, 1959; Dalby, 1968; Hlavaèek, 1982; Slavík, 1992; Kuusk et al., 1996). Results supporting this were obtained in recent studies of epidermal hairs (Celka et al., 2006b) and petals (Celka et al., 2007). The morphological and anatomical variation of *M. alcea* fits within the limits given in descriptions of this species. However, it must be remembered that variation in quantitative traits can greatly exceed the level of genetic variation estimated on the basis of isozymes (Drapikowska and Krzakowa, 2009).

Results of research on DNA polymorphism are comparable with the genetic variation found in this study. Our work revealed a high level of heterozygosity in all populations, however, in most cases F was not statistically significant. Heterozygote excess in populations is not common and therefore has not

been fully explored theoretically. An excess of heterozygotes in populations is explained as overdominant selection favoring heterozygotes (Mitton, 1989), associative overdominance (Nei, 1987) and negative assortative mating (negative selection) (Hartl and Clark, 1989). In our study the Ewens-Waterson neutrality test (Nielsen, 2001) showed lack of selection against homozygotes.

AMOVA revealed high genetic variation within populations and low variation among populations of *M. alcea* (Tab. 5). This is characteristic of vascular plants with a low level of self-fertilization, cross-pollination and wide distribution (Hamrick and Loveless, 1986). An interesting result of these isozyme analyses is the negative inbreeding coefficient in small populations (see Table 2). The percentage of polymorphic loci in the studied populations (P=86.3) is much higher than the mean value reported by Hamrick and Godt (1989) for vascular plants (P=34.2). The level of genetic diversity for *M. alcea* (F_{ST}=0.126) is lower than the value reported by those authors for species with regional distributions (F_{ST}=0.216) and lower than for insect-pollinated and self-pollinated species (F_{ST}=0.216). It is also much higher than in monocotyledons; for example, in *Calamagrostis arundinacea* it reaches F_{ST}=0.0565 (Krzakowa and Dunajski, 2007). In the conifer *Pinus sylvestris* the G_{ST} values ranged from 0.003 to 0.076, and in *P. mugo* G_{ST} ranged from 0.007 to 0.033 (Odrzykoski 2002).

The population groups distinguished in Figures 2 and 3 are only partly consistent with the distinguished geographic regions. The Mantel test showed no significant correlation between genetic and geographic distance (r=0.32, p>0.05). It seems that geographic distance is less important than provenance in explaining the observed diversity of *M. alcea*. Our study showed a lack of correlation between genetic distance and the geographic location of populations. This lack of correlation may be due to the different origins of individual populations. Some may be remnants of old cultivation, while others may have arisen from the past and contemporary spread of *M. alcea* at sites in and near former settlements, in ruderal habitats and along transportation routes. In consequence, geographically close populations can differ genetically. We observed high variation in allele frequencies at the analyzed loci, but no alleles were distinguished as characteristic of individual populations.

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APPENDIX 1. Localities and habitats of the studied populations of *Malva alcea*

No. of pop.	Locality/Region	Geographical location	Habitat	Pop. size
1	Daleszyn 1 (Wielkopolskie, Poland)/II	N 51°56`00.4`` E 17°00`05.1``	cone-shaped mound of late medieval earthwork	80
2	Daleszyn 2 (Wielkopolskie, Poland)/II	N 51°55`46.1`` E 17°00`05.2``	embankments of early medieval rampart	2
3	Dusina (Wielkopolskie, Poland)/II	N 51°55`17.7`` E 17°01`15.2``	cone-shaped mound of early medieval earthwork	40
4	Gostyń (Wielkopolskie, Poland)/II	N 51°55`10.8`` E 17°00`51.7``	roadside ditch	15
5	Sławsko (Zachodniopomorskie, Poland)/II	N 54°23`13.9`` E 16°42`50.6``	wasteland near medieval rampart	45
6	Sławsko (Zachodniopomorskie, Poland)/II	N 54°23`21.7`` E 16°42`48.2``	roadside near medieval rampart	5
7	Bartodzieje (Dolnośląskie, Poland)/II	N 51°41`02.6`` E 16°19`37.3``	roadside	45
8	Karzec (Wielkopolskie, Poland)/II	N51°45`05.2`` E16°53`25.4``	roadside	8
9	Tum near Łęczyca (Łódzkie, Poland)/II	N 52°03`12.5`` E 19°13`40.7``	valley of early medieval rampart	40
10	Koziegłowy (Wielkopolskie, Poland)/II	N 52°26`25.1`` E 16°58`55.3``	roadside. roadside ditch	30
11	Ostrowite-Napole (Kujawsko-Bydgoskie, Poland)/III	N 53°08`46.5`` E 18°57`03.7``	embankments of early medieval rampart	40
12	Łekno (Wielkopolskie, Poland)/II	N 52°50`24.4`` E 17°17`25.7``	embankments of early medieval rampart	50
13	Dowspuda (Podlaskie, Poland)/III	N 53°57`32.9`` E 22°49`18.8``	roadside, roadside ditch	100
14	Płoski on the Narew (Podlaskie, Poland)/III	N 52°54`13.1`` E 23°13`58.5``	roadside near a bridge on Narew River	3
15	Wirów near Drohiczyn on the Bug (Podlaskie, Poland)/III	N 52°26`35.7`` E 22°32`12.3``	cemetery (roadside, roadside ditch)	30
16	Nowosielec (Podkarpackie, Poland)/III	N 50°25`23.5`` E 22°07`54.8``	roadside ditch on forest edge	30
17	Bliżyn (Świętokrzyskie, Poland)/II	N 51°06`51.6`` E 20°44`31.0``	roadside ditch	3
18	Wyszyna Machorowska (Świętokrzyskie, Poland)/II	N 51°09`13.0`` E 20°10`52.6``	roadside, roadside ditch	40
19	Pątnów (Łódzkie region, Poland)/II	N 51°08`51.1`` E 18°36`29.9``	roadside ditch	30
20	Mojęcice (Dolnośląskie, Poland)/II	N 51°17`28.0`` E 16°35`41.4``	roadside, roadside ditch	40
21	Rapaty (Warmińsko-Mazurskie, Poland)/III	N 53°43`53.6`` E 20°09`29.3``	roadside, roadside ditch	40
22	Feldberg (Meclenburg-Vorpommern, Germany)/I	N53°20`34.1`` E13°27`29.0``	roadside	4
23	Fürstenwerder (Meclenburg-Vorpommern, Germany)/I	N53°22`36.4`` E13°34`06.5``	roadside, roadside ditch	40
24	Teterow (Meclenburg-Vorpommern, Germany)/I	N53°47`22.6`` E12°35`52.3``	meadow on island near embankments of early medieval rampart	50

Explanations: I – western region; II – central region; III – eastern region