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Variability of *Anthoxanthum* species in Poland in relation to geographical-historical and environmental conditions: isozyme variation

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Abstract: Variation of 9 isozyme systems was studied in Polish populations of 3 species of the genus *Anthoxanthum*: the native *A. odoratum* s. str. L. and *A. alpinum* Á. Löve & D. Löve, as well as the alien *A. aristatum* Boiss. Results of this study show that *A. odoratum* is characterized by a high isozyme variability of lowland populations, weakly correlated with habitat type, and partial genetic distinctness of montane populations. Moreover, 5 isozyme markers have been identified (*Pgi*-2, *Dia*-2, *Mdh*, *Idh, Pgm*) for the allopolyploid *A. odoratum*. Populations of *A. aristatum* are highly polymorphic (*P* = 98%). The observed isozyme differentiation of its populations (F_{ST} = 0.087**)** is low and gene flow between them (N_m = 5.314) is high. The genetic variation reflects environmental variation only to a small extent and is not significantly related to the phase of chorological expansion of this species. Altitudinal vicariants, *A. alpinum* and *A. odoratum*, are characterized by morphological and isozymatic distinctness, indicating their reproductive isolation. In populations of *A. alpinum*, polymorphism is high (*P* = 76.92%), differentiation among populations is moderate (F_{ST} = 0.198), and gene flow between populations along the altitudinal transect (*Nm* = 1.709) is relatively low.

Key words: Poaceae, *Anthoxanthum alpinum*, *Anthoxanthum aristatum*, *Anthoxanthum odoratum*, Poland, isozymes, genetic differentiation

Contents

1. Introduction

The genus *Anthoxanthum* L. in the Polish flora is represented by *A. odoratum* L. s. str., *A. alpinum* Á. Löve & D. Löve, and *A. aristatum* Boiss. (Mirek *et al.* 2002). Till the mid-20th century, only 2 species of this genus were distinguished in Poland: *A. odoratum* and *A. aristatum*. Later on, cytological analysis of Polish specimens of *A. odoratum* showed that, like in other parts of Europe, also in Poland *A. odoratum* is a collective species and includes 2 cytotypes: the diploid *A. alpinum* ($2n = 10$), whose distribution is limited to the subalpine and alpine zones, and the tetraploid *A. odoratum* ($2n = 20$), found in lowlands and at lower altitudes in mountains, below the forest line (Rozmus 1958).

Isozyme analysis is particularly useful for distinguishing between diploids and auto- and allopolyploids in homologous taxa (Oja & Jaaska 1998; Zeidler 2000; Badr *et al.* 2002; Nyberg Berlund *et al.* 2006; Krzakowa & Dunajski 2007; Angelov & Ivanova 2012). Allelic variants of enzymes have been analysed by electrophoresis since the 1970s, to investigate the genetic polymorphism of large numbers of organisms. Isozymes are favourable as genetic markers because of their codominant nature, which makes it possible to distinguish homozygotes from heterozygotes. On the basis of data collected in this way, genetic parameters of population structure can be calculated.

This study was aimed: (*i*) to analyse genetic variation between populations of the native *A. odoratum*, representing various phases of ecological expansion within its natural range; (*ii*) to determine if there are any significant genetic differences between populations of *A. aristatum* representing various phases of chorological expansion, i.e. outside the natural limits of its distribution; (*iii*) to assess genetic variation between samples of *A. odoratum* and *A. alpinum* collected along the altitudinal transect in the Babia Góra massif; (*iv*) to determine if there are any hybrids in the contact zone of both species; (*v*) to determine if there are any correlations between morphological and isozyme variation of the studied species.

2. Material and methods

2.1. Material

Plant material was collected from various parts of Poland in 2007-2011 during the flowering and fruiting of the studied grass species: *Anthoxanthum odoratum*, *A. alpinum*, and *A. aristatum* (Fig. 1, Appendix 1). The collected seeds were used to establish a plantation in the Botanic Garden of the Adam Mickiewicz University in Poznań, Poland. Caryopses of *A. odoratum* and *A. aristatum* were sown in pots filled with parboiled garden soil and kept in a greenhouse. Next the seedlings were transplanted, each to a separate pot. When the plants reached the 3-leaf stage, they were planted outdoors. Plots of the 2 species were spatially isolated to prevent interspecific hybridization. Next, from individual populations, material was collected for isozyme analyses.

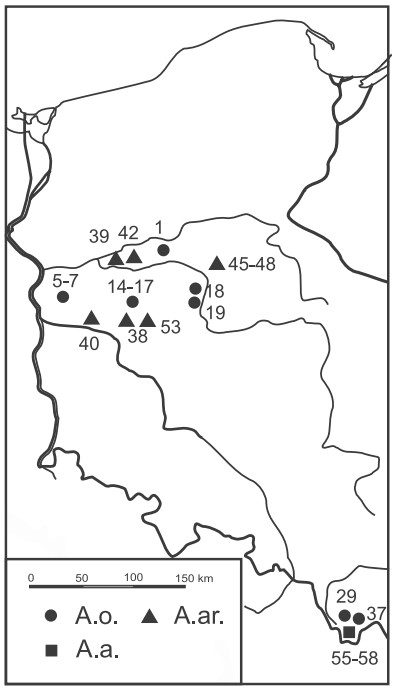

Fig. 1. Sample collection sites of *Anthoxanthum odoratum*, *A. alpinum* and *A. aristatum* in Poland
Explanations: A.o. – *A. odoratum*, A.a. – *A. alpinum*, A. ar. – *A. aristatum*; site numbers – see Appendix 1

Table 1. Enzyme Commission numbers (Enzyme 2010), buffer systems used for separation of isozymes, their quaternary structure (Wendel & Wendel 1989), and interpretation of gel zymograms of *Anthoxanthum odoratum*, *A. alpinum*, and *A. aristatum*

Buffer	Enzyme	E.C. number	Quaternary structure	*A. aristatum* locus	Allele	*A. alpinum* locus	Allele	*A. odoratum* Band locus	Band
A	PGI	5.3.1.9	dimer	*Pgi*-1	1,2	*Pgi*-1	1,2	*Pgi*-1	1,2,3
				Pgi-2	1,2,3,4	*Pgi*-2	1	*Pgi*-2	1,2,3,4,5
	PRX	1.11.1.7	dimer	*Px*-1	1,2	*Px*-1	1,2,3	*Px*-1	1,2,3,4,5
				Px-2	1,2	*Px*-2	1	*Px*-2	1,2,3
	GOT	2.6.1.1	dimer	*Got*-1	1,2	*Got*-1	1,2	*Got*-1	1,2,3
				Got-2	1,2	*Got*-2	1,2	*Got*-2	1,2,3
	DIA	1.8.1.4	monomer	*Dia*-1	1,2	*Dia*-1	1,2	*Dia*-1	1,2
				Dia-2	1,2	*Dia*-2	1,2,3,4	*Dia*-2	1,2,3
B	IDH	1.1.1.42	dimer	*Idh*	1,2	*Idh*	1,2	*Idh*	1,2,3,4,5
	PGM	5.4.2.2	monomer	*Pgm*	1,2,3	*Pgm*	1,2,3	*Pgm*	1,2,3
	SDH	1.1.1.25	monomer	*Sdh*	1,2	*Sdh*	1,2	*Sdh*	1,2
	MDH	1.1.1.37	dimer	*Mdh*	1,2,3	*Mdh*	1,2,3,4	*Mdh*	1,2,3,4,5
	PGD	1.1.1.44	dimer	*Pgd*	1,2,3	*Pgd*	1,2	*Pgd*	1,2,3

Material for population analyses of *A. alpinum* was collected in the field, in the Babia Góra massif in the south of Poland.

Isozyme analysis was performed for 12 populations of *A. odoratum*, 9 of *A. aristatum*, and 4 of *A. alpinum*. The analysis involved a total of 550 plants, usually 10-32 from individual populations except for a population of *A. alpinum* (no. 58), which was very small, so only 6 plants from that population were studied.

2.2. Genetic methods

Leaves of individual plants were placed in separate paper bags, next labelled (specimen no., population no.), and then transported in a portable refrigerator at about 10°C. Variability was assessed in 15 enzyme systems (Enzyme 2010), and 9 polymorphic isozymes were selected for further genetic research: phosphoglucoisomerase (PGI, EC 5.3.1.9.), isocitrate dehydrogenase (IDH, EC 1.1.1.42), peroxidase (PX, EC 1.11.1.7), glutamate oxaloacetic transaminase (GOT, EC 2.6.1.1), NADH diaphorase (DIA, EC 1.8.1.4), phosphoglucomutase (PGM, EC 5.4.2.2), shikimate dehydrogenase (SHD, EC 1.1.1.25), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), and malate dehydrogenase NAD^+ (MDH, EC 1.1.1.37).

Leaf samples (about 50 mg each) were homogenized with 80 µl of extraction buffer (Gottlieb 1981) or of distilled water for analysis of peroxidases (Szweykowski & Odrzykoski 1990; Krzakowa 1996). Proteins were separated electrophoretically on 10% starch gel. For PGI, PX, GOT, and DIA, buffer A was used, composed of Tris-citrate (pH 8.2) and lithium borate (pH 8.3), whereas for IDH, PGM, SDH, MDH, and PGD, buffer B was used, i.e. morpholine-citrate (pH 6.1), gel buffer was prepared by dilution of electrode buffer ratio 1:14. After separation, the enzymes were stained using standard methods (Wendel & Weenden 1989).

Isozyme loci were labelled using 3-letter abbreviations of enzyme names. Whenever a larger number of isozymes was detected, the abbreviation was followed by successive numbers (e.g. *Pgi*-1 is the name of the fastest migrating isozyme). Individual alleles in the given locus were numbered sequentially, too. Genetic interpretation followed the rules presented by Wendel and Weeden (1989), on the basis of information on quaternary structure of individual enzymes (Table 1).

Ploidy level in samples collected along an altitudinal transect on Babia Góra was measured by flow cytometry (Śliwińska 2008; Kubešová *et al.* 2010) in Kutnowska Hodowla Buraka Cukrowego Ltd. in Straszkowo, Poland.

2.3. Statistical analysis

In diploid species (*A. aristatum* and *A. alpinum*), the following genetic parameters were determined: allele frequencies, effective number of alleles per locus (Kimura & Crow 1964), proportion of polymorphic loci, observed heterozygosity (i.e. recorded frequency of heterozygotes in the population), and expected heterozygosity (i.e. frequency of heterozygotes under Hardy-Weinberg equilibrium). Observed and expected heterozygosity were used to calculate the inbreeding coefficient F to determine the mating system. Deviations from Hardy-Weinberg equilibrium were analysed statistically by chi-square (χ^2) test. The F-statistics helped to determine how genetic variation was distributed within and between populations. Gene flow (N_m) between populations was calculated from the formula: $N_m = 0.25\times(1\text{-}F_{ST})/F_{ST}$, where F_{ST} denotes genetic differentiation (i.e. standardized variance in allele frequencies between populations: 0 when frequencies are identical, 1 when the populations do not share any genetic diversity) (Wright 1951). Statistical significance of F_{ST} was analysed using the chi-square test. To determine the

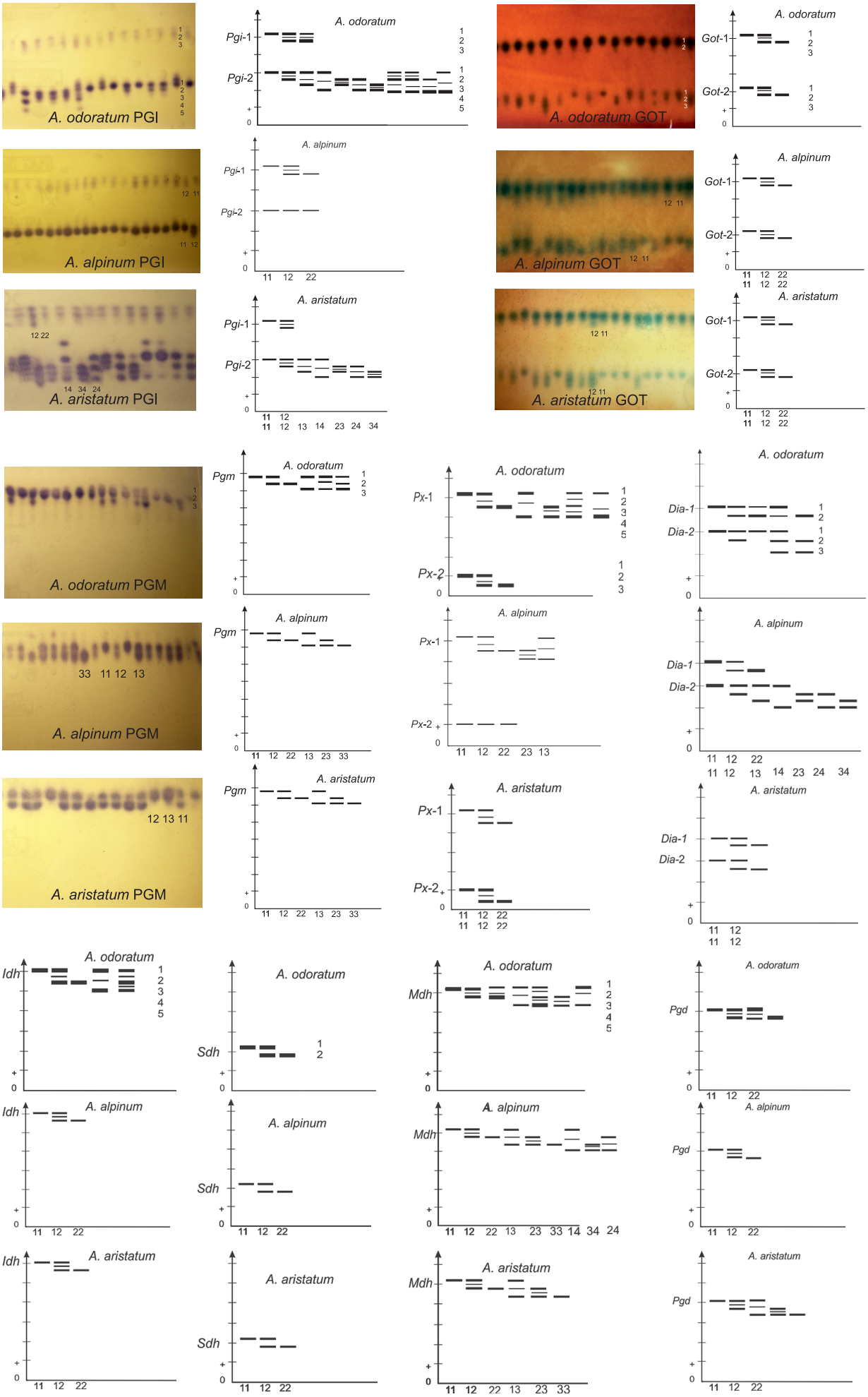

Fig. 2. Interpretation of gel zymograms of phosphoglucoisomerase (PGI), glutamate oxaloacetic transaminase (GOT), phosphoglucomutase (PGM), peroxidase (PX), NADH diaphorase (DIA), isocitrate dehydrogenase (IDH), shikimate dehydrogenase (SDH), malate dehydrogenase NAD^+ (MDH), and 6-phosphogluconate dehydrogenase (PGD) loci in *Anthoxanthum odoratum*, *A. alpinum*, and *A. aristatum*

effect of selection on individual loci, Ewens-Watterson homozygosity test of neutrality was used (1000 permutations) (Manly 1985). Moreover, Nei's (1978) unbiased genetic distances were calculated. The distances formed a basis for dendrogram construction using UPGMA (unweighted pair group method with arithmetic mean) and principal component analysis (PCA). Hierarchic molecular analysis of variance (AMOVA) was used to assess the genetic structure of populations (Excoffier *et al.* 1992). Additionally, potential imbalance of linkage between loci was tested (i.e. nonrandom association of alleles at linked loci). Negative assortative mating was tested using GenAlex 6.3 software (Peakall & Smouse 2006). Also Popgene (Yeh & Boule 2000) and Statistica 8.0 for Windows software were used for calculations. To determine if Nei's genetic distances are correlated with Mahalanobis distances based on biometric data, Spearman correlation coefficient was calculated (Lange 1995). Variability of *A. odoratum* was analysed on the basis of phenotypes of individual isozymes (Fig. 2). This method is used for allopolyploid species (Abha *et al.* 2006). Binary data were generated on the basis of presence or absence of a band at a given locus. Genetic similarity between populations was estimated on the basis of Jaccard similarity coefficient, from the formula:

$$S_{AB}=N_{AB}/(N_A+N_B-N_{AB}),$$

where N_A = number of alleles in genotype A; N_B = number of alleles in genotype B; N_{AB} = number of alleles in genotypes A and B. Next, a dendrogram was constructed using UPGMA. PCA based on band frequency was used to estimate the pattern of variation among populations. The above analyses were made using NTSYS-pc software.

To assess the correlation between the recorded morphological and isozyme variation, Spearman rank correlation coefficients (Lange 1995) between Nei's (1978) genetic distances and Mahalanobis distances based on results of morphological analyses for populations of *A. odoratum*, *A. alpinum*, and *A. aristatum* were calculated (Drapikowska 2013). Additionally, a canonical discriminant analysis was performed on the basis of morphological data for populations of all 3 species (Sneath & Sokal 1973; Drapikowska 2013).

3. Results

3.1. Isozyme variation of *Anthoxanthum odoratum*

Samples of this species were analysed in respect of variation of 9 enzyme systems (Table 1). The gel zymograms for individual polymorphic isozymes are presented in Fig. 2. For each enzyme, phenotypic patterns of the studied species were compared, to identify isozyme markers that can be used to distinguish tetraploid individuals of *A. odoratum* from diploid individuals of *A. aristatum* and *A. alpinum*.

Phosphoglucoisomerase (PGI)

Activity of the dimeric PGI was detected in 2 regions of the anodal part of the gel. Two loci were found: *Pgi*-1 and *Pgi*-2. Isozyme *Pgi*-1 was composed of 1 or 3 bands. In the 3-band phenotype, the most conspicuous were the 2 extreme bands: the fastest and the slowest one. Phenotypes of *Pgi-2* were stained much less, and consisted of 1, 3, or 5 bands, stained evenly.

On the basis of PGI phenotype, *A. odoratum* can be distinguished from its diploid relatives: *Pgi*-2 in *A. odoratum* has 1, 3 or 5 bands, whereas *A. aristatum* has an additional, 4th allele at locus *Pgi-2*, and *A. alpinum* has only 2 alleles at locus *Pgi-2*.

Isocitrate dehydrogenase (IDH)

In the IDH system (dimer), one locus *Idh* was discovered in the anodal part of the gel. Individuals with 1-, 3-, and 5-band phenotypes were found. Among 3- and 5-band phenotypes, differences in band intensity were observed. This is associated with quaternary structure of dimeric enzymes, whose heterozygotes have a 3-band phenotype. Additionally, in allotetraploid plants, some bands are doubled and visible as one, more intensive band. On the basis of IDH phenotype, *A. odoratum* can be distinguished, as it has additional 4th and 5th bands, whereas diploid species have only 2 alleles at this locus.

Peroxidase (PX)

Dimeric PX were represented by 2 loci in the anodal part of the gel: *Px*-1 and *Px*-2. At *Px*-1, the analysed specimens had 1-, 3- or 5-band phenotypes, while *Px*-2 had 1 or 3 bands. Dimeric PX was first analysed in rice (*Oryza sativa*, Shahi *et al.* 1969) and next in common reed (*Phragmites australis*, Krzakowa 1996).

NADH diaphorase (DIA)

Activity of monomeric DIA was detected in 2 regions of the anodal part of the gel. At *Dia*-1, the analysed specimens had 1-2 bands, whereas at *Dia*-2, they had 1-3 bands. Among 2- and 3-band specimens, differences in band intensity were observed. This is due to co-occurrence of bands of various alleles in allopolyploids. On the basis of DIA phenotype *A. odoratum* can be distinguished from its diploid relatives.

Glutamate oxaloacetic transaminase (GOT)

Activity of the dimeric GOT was detected in 2 regions of the anodal part of the gel and marked as *Got-1* and *Got*-2. Phenotypes were composed of 1 or 3 bands, with varying intensity.

Phosphogluconate dehydrogenase (PGD)

For this enzyme, one locus *Pgd* was discovered in the anodal part of the gel. It was represented by 1- and 3-band phenotypes with varying band intensity. In

Table 2. Bands frequency in *Anthoxanthum odoratum* populations

Locus	Bands	7MM	5FR	14PF	15MM	17FR	1PF	6FR	18pG	16EF	19PP	37LM	29LM
Pgi-1	1	0.733	0.800	0.935	0.900	0.900	0.833	1.000	0.733	0.870	0.833	0.727	0.905
	2	0.733	0.567	0.968	0.933	0.500	0.833	0.400	0.400	0.400	0.367	0.455	0.429
	3	0.367	0.233	0.129	0.300	0.633	0.667	0.600	0.800	0.533	0.700	0.545	0.571
Pgi-2	1	0.933	1.000	0.290	0.167	0.100	0.567	0.000	0.200	0.533	0.167	0.727	0.810
	2	0.300	0.333	0.452	0.267	0.000	0.133	0.600	0.400	0.000	0.100	0.273	0.190
	3	0.733	0.767	0.839	0.767	0.900	1.000	0.933	0.933	0.833	0.933	0.818	0.619
	4	0.833	0.233	0.419	0.867	0.833	1.000	0.467	0.067	0.900	0.600	0.636	0.714
	5	0.433	0.233	0.290	0.167	0.100	0.567	0.000	0.200	0.533	0.167	0.273	0.238
Idh-1	1	0.700	0.800	0.806	0.800	0.933	0.533	0.667	0.867	0.967	0.500	1.000	0.952
	2	0.733	0.567	0.226	0.233	0.133	0.533	0.333	0.133	0.033	0.500	1.000	0.857
	3	0.333	0.267	0.516	0.633	0.867	0.600	0.667	0.867	0.900	0.600	0.545	0.143
	4	0.833	0.867	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.727	0.667
	5	0.267	0.267	0.065	0.133	0.133	0.133	0.333	0.200	0.133	0.067	0.455	0.667
Px-1	1	0.500	0.700	0.355	0.800	0.400	0.533	0.867	0.600	0.433	0.600	0.455	0.429
	2	0.933	0.967	0.935	0.567	0.467	0.867	0.200	0.400	0.433	0.767	0.455	0.429
	3	0.300	0.433	0.871	0.633	0.933	0.633	0.200	0.600	0.733	0.700	0.364	0.571
	4	0.600	0.533	0.258	0.367	0.167	0.467	0.267	0.267	0.267	0.500	0.636	0.381
	5	0.467	0.833	1.000	1.000	0.867	1.000	1.000	1.000	0.733	0.733	0.727	0.667
Px-2	1	0.500	0.767	0.290	0.200	0.333	0.567	0.133	0.133	0.467	0.967	0.909	0.667
	2	0.767	0.233	0.806	0.867	0.267	0.767	0.600	0.867	0.700	0.733	1.000	0.952
	3	1.000	1.000	0.452	0.667	0.967	0.667	0.467	0.800	0.933	0.967	1.000	0.952
Dia-1	1	0.267	0.300	0.968	0.933	0.900	1.000	1.000	0.867	0.900	0.800	0.545	0.952
	2	0.633	0.667	0.387	0.300	0.833	0.600	0.067	0.133	0.433	0.467	0.818	0.905
Dia-2	1	0.600	0.833	0.742	0.733	1.000	0.533	0.533	0.933	0.700	0.567	0.818	0.905
	2	0.967	1.000	0.968	0.967	0.900	0.600	0.933	1.000	0.933	0.600	0.545	0.429
	3	0.233	0.333	0.742	0.600	0.067	0.767	0.800	0.800	0.767	0.867	0.727	0.952
Got-1	1	1.000	1.000	0.968	0.367	0.967	0.667	0.067	0.400	0.900	0.600	0.636	0.714
	2	0.667	0.567	0.419	0.800	0.700	0.600	0.333	0.867	0.767	0.567	0.545	0.333
	3	0.133	0.167	0.645	0.167	0.400	0.333	0.733	0.600	0.433	0.567	1.000	1.000
Got-2	1	0.467	0.333	1.000	1.000	0.733	0.967	0.933	1.000	0.700	0.733	1.000	0.714
	2	0.433	0.433	0.161	0.067	0.967	1.000	0.533	0.333	0.900	0.967	0.364	0.476
	3	0.533	0.433	1.000	1.000	0.733	0.967	0.933	1.000	0.833	0.633	0.545	0.429
Pgd	1	1.000	0.967	0.161	0.067	0.933	1.000	0.533	0.400	0.733	0.967	0.818	0.714
	2	0.700	0.900	0.161	0.067	0.933	1.000	0.600	0.400	0.733	0.967	0.364	0.429
	3	0.333	0.742	0.600	0.067	0.767	0.800	0.333	0.767	0.867	0.727	0.730	0.429
Mdh	1	1.000	0.133	0.533	0.867	0.633	0.467	1.000	0.567	0.440	0.111	0.150	0.571
	2	0.267	0.430	0.670	0.290	0.670	0.267	0.300	0.340	0.690	0.456	0.570	0.381
	3	0.467	0.700	0.933	0.900	0.067	0.133	0.433	0.467	0.818	0.680	0.560	0.667
	4	0.967	0.935	0.567	0.467	0.867	0.200	0.400	0.433	0.767	0.455	0.429	0.900
	5	0.433	0.871	0.633	0.933	0.633	0.200	0.600	0.733	0.700	0.364	0.571	0.433
Pgm	1	0.467	1.000	0.567	0.767	0.667	1.000	0.600	0.230	0.350	0.680	0.280	0.700
	2	0.900	1.000	1.000	0.867	0.900	0.800	0.545	0.267	0.298	0.267	0.180	0.933
	3	0.833	0.600	0.067	0.133	0.433	0.467	0.818	0.670	0.140	0.170	0.470	0.767
Sdh	1	1.000	0.533	0.533	0.933	0.700	0.567	0.818	0.300	0.400	0.360	0.120	0.900
	2	1.000	0.133	0.533	0.867	0.633	0.467	1.000	0.567	0.780	0.550	0.130	0.767

the diploid *A. aristatum*, an additional, 3rd allele was discovered at this locus, which makes it possible to distinguish the two species.

Malate dehydrogenase NAD^+ (MDH)

Activity of the dimeric MDH was detected at one locus *Mdh*, with 1-, 3-, and 5-band phenotypes, varying in band intensity. On the basis of MDH phenotype *A. odoratum* can be distinguished from its diploid relatives.

Phosphoglucomutase (PGM)

For the monomeric PGM, one locus was found in the anodal part of the gel. It was represented by 1-, 2-, and 3-band phenotypes with varying band intensity. It is possible to distinguish tetraploid individuals from the diploid *A. aristatum* and *A. alpinum* on the basis of band intensity. Besides, heterozygous specimens of *A. odoratum* can have three bands, while diploids always have two bands.

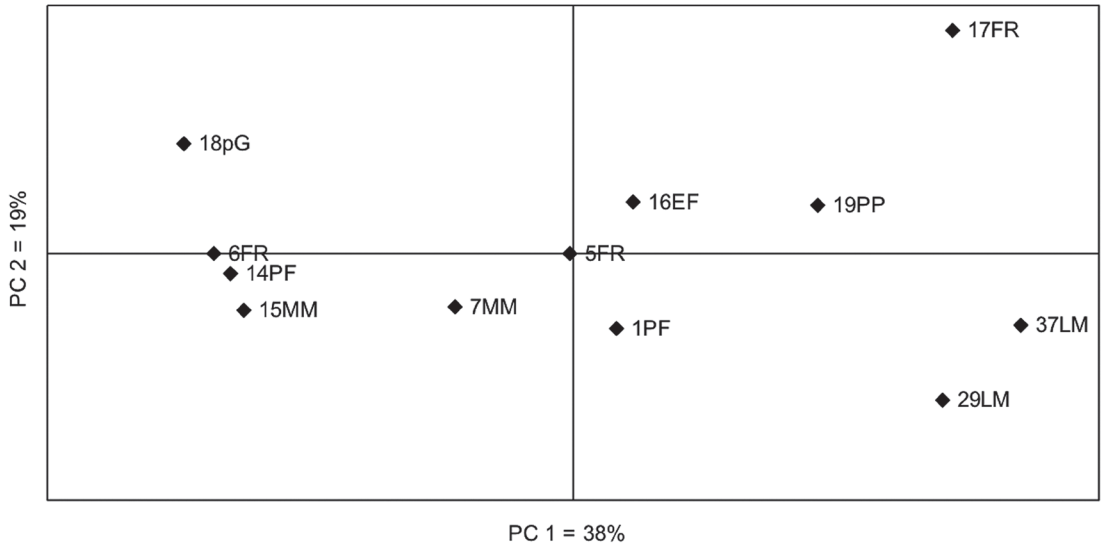

Fig. 3. Distribution of *Anthoxanthum odoratum* populations in the system of the first two principal components (PC1 and PC2) in respect to band frequency for all loci.
Explanations: PF – pine forest, DM – dry meadow, MM – moist meadow, pG – sandy grassland near pine forest plantation, FR – roadside in pine forest, AR – field roadside, EF – moist edge of pine forest, PP – pine forest plantation, LM – lower montane meadow; site numbers – see Appendix 1

Shikimate dehydrogenase (SHD)

For the monomeric SDH, one locus *Shd* was found in the anodal part of the gel. In this region, 1- or 2-band phenotypes with varying intensity were observed.

The band patterns for each enzyme system were used to construct a binary matrix. Band frequency was calculated for each enzyme system (Table 2, Appendix 2). In *A. odoratum*, *Idh*-1-4 bands were most frequent (100%) in 8 populations: 14PF, 15MM, 17FR, 1PF, 6FR, 18pG, 16EF, and 19PP. Another very frequent phenotype was *Px*-1-5, in 5 populations: 14PF, 15MM, 1PF, 6FR, 18pG. In a roadside in pine forest (17AR), in pne forest (14PF) and a lower montane population (29LM), the largest number of very frequent bands (90-100%) were found.

Results of PCA (Fig. 3) show that populations of *A. odoratum* from lower montane meadows (29ML and 37ML) in the Babia Góra massif are close to each other in the diagram and are distinguished by positive values of PCA1. Positive values of PCA1 are recorded also for a population from a pine plantation (19PP) on Nowy Tomyśl Sandur, distinguished also by positive values of PCA2, and a population from a roadside in pine forest (17FR) on Nowy Tomyśl Sandur. The left part of the diagram includes a population from a roadside in pine forest (6FR) in the Rzepin Forest, from pine forest (14PF) and from a moist meadow (15MM) on Nowy Tomyśl Sandur, and a population from sandy grassland (18pG) in Morasko. Some loci (*Idh* 1-2 and 4, *Px*-1-5, *Dia*-1-2, *Got*-2-3, *Pgd*-1 and 3, *Pgm*-2 *Sdh*-1) are strongly correlated with the first 2 principal components: PC1 and PC2 (Table 3).

Genetic similarity between populations was estimated on the basis of Jaccard similarity coefficient. Cluster analysis (Fig. 4, Table 4) separated three groups of individuals. The first group (I) is composed of individuals from all the studied populations, forming several subgroups. It is noteworthy that individuals from the lower montane zone in the Babia Góra massif (37LM and 29LM) are grouped together. The second group (II) is composed of several nearly homogeneous subgroups. The first one consists of 9 individuals from a lower montane population (29LM), from pine forest (14PF) and from a roadside in pine forest (6FR). The next subgroups are composed of individuals from a moist meadow (15MM), moist edge of pine forest (16EF), sandy grassland near pine forest plantation (18pG), and a roadside in pine forest (17FR). The last subgroup includes individuals from only one population (18pG). Group III is distant from the others (I and II) and consists of 2 individuals from a pine plantation (19PP) and single individuals from a lower montane meadow (37LM) and moist meadow (7MM), (Table 3, 4, Fig. 4).

3.2. Isozyme variation of *Anthoxanthum aristatum*

In 9 enzyme systems, 13 loci were found (Table 1). The gel zymograms are presented in Fig. 2.

Phosphoglucoisomerase (PGI)

PGI activity was detected in 2 regions of the anodal part of the gel: loci *Pgi*-1 (with 2 alleles) and *Pgi*-2 (with 4 alleles). At both loci, 1-band phenotypes were observed, corresponding to homozygous genotype, and 3-band phenotypes in heterozygotes. The pattern in characteristic of dimeric enzymes (Lack & Kay 1986).

Table 3. Correlation between bands frequency and the first two principal components (PC1 and PC2) in *Anthoxanthum odoratum* (strongest correlations are marked in bold)

Locus	Allele	PC1	PC2
Pgi-1	1	-0.530	0.166
	2	-0.234	0.583
	3	-0.071	-0.760
Pgi-2	1	0.853	0.274
	2	-0.369	0.470
	3	-0.515	-0.561
	4	0.156	-0.044
	5	0.328	-0.111
Idh-1	1	0.160	-0.043
	2	**0.758**	0.077
	3	-0.637	-0.537
	4	**-0.772**	-0.141
	5	0.498	0.095
Px-1	1	-0.404	0.319
	2	0.361	0.364
	3	-0.236	-0.313
	4	0.700	0.111
	5	**-0.805**	-0.075
Px-2	1	0.807	-0.325
	2	-0.075	-0.151
	3	**0.812**	-0.219
Dia-1	1	-0.699	-0.363
	2	**0.837**	-0.071
Dia-2	1	0.156	-0.064
	2	-0.471	0.486
	3	-0.251	-0.417
Got-1	1	0.605	0.131
	2	-0.185	-0.124
	3	0.056	-0.472
Got-2	1	-0.683	-0.346
	2	0.197	-0.634
	3	**-0.931**	-0.091
Pgd	1	**0.751**	-0.305
	2	0.394	-0.345
	3	0.250	**-0.739**
Mdh	1	-0.449	0.564
	2	0.062	-0.384
	3	-0.093	0.301
	4	0.568	0.428
	5	-0.323	0.378
Pgm	1	0.117	0.317
	2	0.109	**0.750**
	3	0.322	0.238
Sdh	1	-0.063	**0.752**
	2	-0.393	0.348
	3	0.107	-0.744

Isocitrate dehydrogenase (IDH)

In this enzyme system, one locus *Idh-1* was detected in the anodal part of the gel, with 2 alleles. Homozygotes had a single band whereas heterozygotes had 3 bands (IDH is dimeric).

Peroxidase (PX)

Dimeric PX was encoded at two loci: *Px*-1 and *Px*-2 in the anodal part of the gel. Both isozymes had two alleles each. Their phenotypes were composed of 1 or 3 bands, for homo- and heterozygotes, respectively (Fig. 2). In the cathodal part, peroxidase activity was also detected. However, the patterns were not clear enough to allow their analysis.

NADH diaphorase (DIA)

Activity of monomeric DIA was detected in 2 regions of the anodal part of the gel. Loci *Dia*-1 and *Dia*-2 had 2 alleles each. *A. aristatum* had 1- and 2-band phenotypes, corresponding to homo- and heterozygous genotypes, respectively.

Glutamate oxaloacetic transaminase (GOT)

Activity of this enzyme was detected in 2 regions of the anodal part of the gel and labelled as *Got*-1 and *Got*-2. Both isozymes had 2 alleles each. Phenotypes were characteristic of dimeric enzymes: a single band for homozygotes and 3 bands for heterozygotes.

Phosphogluconate dehydrogenase (PGD)

In the PGD system, one locus *Pgd* was found in the anodal part of the gel, with 3 alleles. Phenotypes were characteristic of dimeric enzymes.

Malate dehydrogenase NAD^+ (MDH)

Activity was detected at one locus *Mdh* with 3 alleles in the anodal part of the gel. Homozygotes had a single band, while heterozygotes had 3 bands (MDH is dimeric).

Phosphoglucomutase (PGM)

In the PGM system, one locus was found in the anodal part of the gel, with 3 alleles. Phenotypes were characteristic of monomeric enzymes.

Shikimate dehydrogenase (SHD)

One locus *Shd* was detected in the anodal part of the gel, with 2 alleles. Phenotypes were characteristic of monomeric enzymes: a single band for homozygotes and 2 bands for heterozygotes.

In the 9 analysed enzyme systems, 13 loci and 31 alleles were detected (Table 5). For each locus, alleles and genotypes were counted and their frequency was calculated. The differences are significant (χ^2 test). Allele frequency varied widely between the enzyme systems. *Pgd* allele 1 was most frequent, with a mean frequency of 0.832. Rare alleles, whose frequency is equal to or lower than 0.05, were found at 2 loci: *Pgd* allele 2 and 3 and *Pgm*-3 in population 42A and *Mdh* allele 3 in populations 38A and 42A (Table 5). Population 42A from an arable field in the Noteć Forest is distinguished by the presence of private alleles at 2 loci: *Pgd* and *Pgm* (Fig. 5, Table 6).

Mean number of alleles per locus (*A*) varied from 2.00 in population 40F to 2.38 in population 42A.

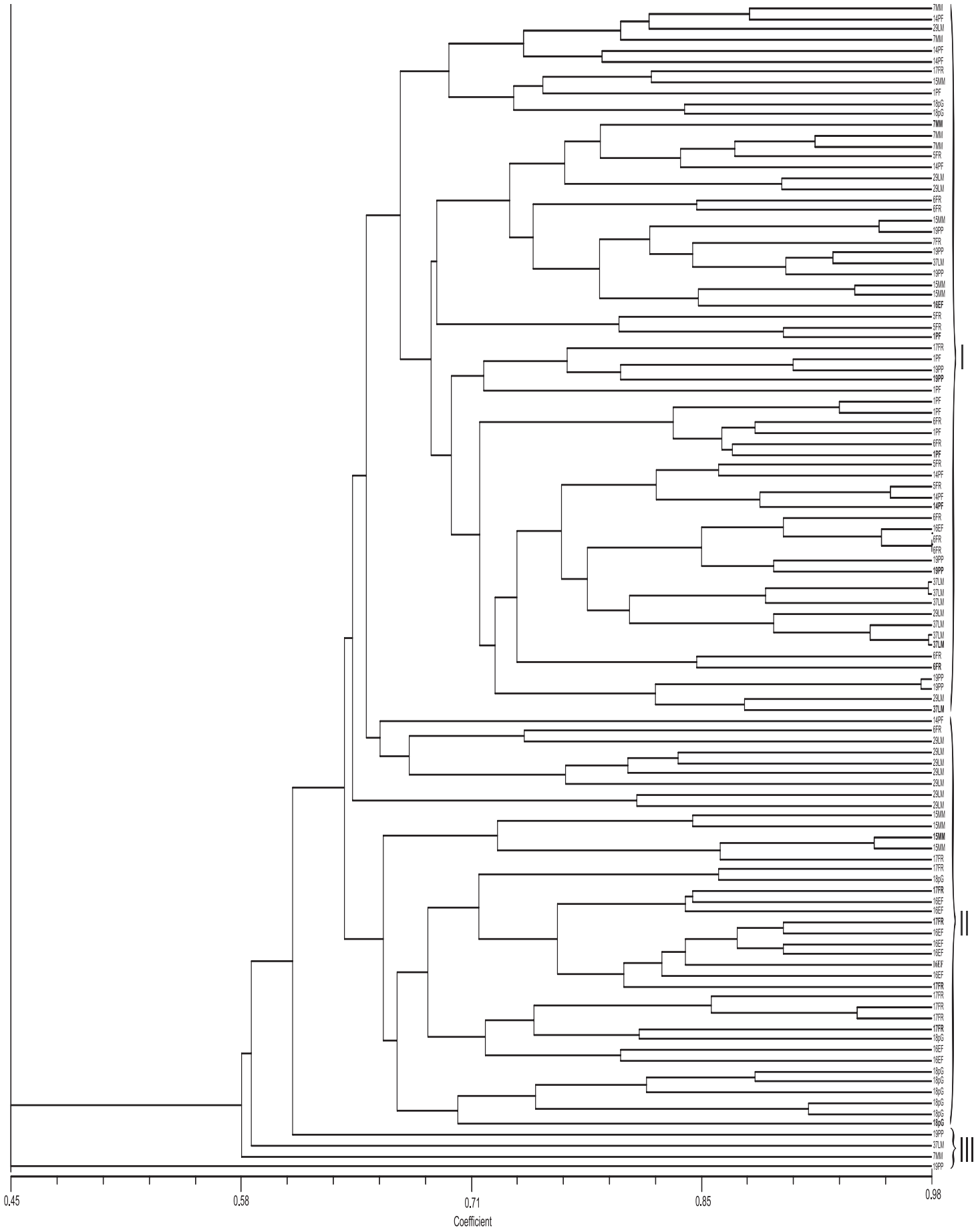

Fig. 4. UPGMA dendrogram based on Jaccard similarity coefficients for 12 *Anthoxanthum odoratum* populations
Explanations: PF – pine forest, DM – dry meadow, MM – most meadow, pG – sandy grassland near pine forest plantation, FR – roadside in pine forest, AR – field roadside, EF – edge of pine forest, PP – pine forest plantation, LM – lower montane meadow; site numbers – see Appendix 1; I-III – main groups of populations

Table 4. Individuals from mountain populations of *Anthoxanthum odoratum*, arranged in the order of corrections to particular groups in cluster analysis – see Fig. 4

Cluster	Specimens
I	7MM, 14PF, 29LM, 7MM, 14PF, 14PF, 17FR, 15MM, 1PF, 18pG, 18pG, 7MM, 7MM, 7MM, 5FR, 14PF, 29LM, 29LM, 6FR, 6FR, 15MM, 19PP, 7FR, 19PP, 37LM, 19PP, 15MM, 15MM, 16EF, 5FR, 5FR, 1PF, 17FR, 1PF, 19PP, 19PP, 1PF, 1PF, 1PF, 6FR, 1PF, 6FR, 1PF, 5FR, 14PF, 5FR, 14PF, 14PF, 6FR, 16EF, 6FR, 6FR, 19PP, 19PP, 37LM, 37LM, 37LM, 29LM, 37LM, 37LM, 37LM, 6FR, 6FR, 19PP, 19PP, 29LM, 37LM
II	14PF, 6FR, 29LM, 29LM, 29LM, 29LM, 29LM, 29LM, 29LM, 15MM, 15MM, 15MM, 15MM, 17FR, 17FR, 18pG, 17FR, 16EF, 16EF, 17FR, 16EF, 16EF, 16EF, 16EF, 16EF, 17FR, 17FR, 17FR, 17FR, 17FR, 18pG, 16EF, 16EF, 18pG, 18pG, 18pG, 18pG, 18pG, 18pG
III	19PP, 37LM, 7MM, 19PP

Effective number of alleles per locus (N_e) ranged from 1.677 in population 38A to 1.863 in population 39pG (Fig. 5, Table 7). Populations of *A. aristatum* were highly polymorphic. In 5 populations all the analysed loci were polymorphic, whereas in the others, *P* was close to 90%. Observed heterozygosity (H_o) varied from 0.385 in population 48aG to 0.517 in population 46fG. In most populations, deviations from the Hardy-Weinberg equilibrium were noticed. Mean values of inbreeding coefficient (*F*) were positive in 2 populations (48aG and 42A), indicating a small excess of homozygotes in these populations. In the others, *F* values were negative (low-

Table 5. Allele frequency in *Anthoxanthum aristatum* populations

Locus	Allele	39pG	38A	45A	42A	40F	46fG	47AR	53aG	48aG	Mean
Pgi-1	1	0.769	0.800	0.817	0.783	0.750	0.639	0.850	0.750	0.797	0.773
	2	0.231	0.200	0.183	0.217	0.250	0.361	0.150	0.250	0.203	0.227
Pgi-2	1	0.135	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.016	0.026
	2	0.423	0.300	0.450	0.233	0.450	0.194	0.433	0.467	0.469	0.380
	3	0.231	0.550	0.400	0.400	0.450	0.528	0.400	0.150	0.297	0.378
	4	0.212	0.150	0.150	0.283	0.100	0.278	0.167	0.383	0.219	0.216
Idh	1	0.692	0.800	0.867	0.600	0.750	0.833	0.783	0.617	0.813	0.751
	2	0.308	0.200	0.133	0.400	0.250	0.167	0.217	0.383	0.188	0.250
Px-1	1	0.404	0.500	0.417	0.483	0.350	0.833	0.600	0.600	0.609	0.533
	2	0.596	0.500	0.583	0.517	0.650	0.167	0.400	0.400	0.391	0.467
Px-2	1	0.731	0.750	0.717	0.600	0.700	0.222	0.383	0.633	0.453	0.577
	2	0.269	0.250	0.283	0.400	0.300	0.778	0.617	0.367	0.547	0.423
Dia-1	1	0.596	0.867	0.733	0.833	0.850	0.639	0.783	0.867	0.656	0.758
	2	0.404	0.133	0.267	0.167	0.150	0.361	0.217	0.133	0.344	0.242
Dia-2	1	0.865	0.883	0.767	0.350	0.650	0.667	0.583	0.700	0.609	0.675
	2	0.135	0.117	0.233	0.650	0.350	0.333	0.417	0.300	0.391	0.325
Got-1	1	0.596	0.733	0.367	0.233	1.000	0.611	0.800	0.833	0.859	0.670
	2	0.404	0.267	0.633	0.767	0.000	0.389	0.200	0.167	0.141	0.330
Got-2	1	0.615	0.633	0.417	0.133	0.550	0.778	0.633	0.933	0.813	0.612
	2	0.385	0.367	0.583	0.867	0.450	0.222	0.367	0.067	0.188	0.388
Pgd	1	0.769	1.000	0.633	0.933	0.800	0.778	0.883	0.817	0.875	0.832
	2	0.231	0.000	0.367	0.033	0.200	0.222	0.117	0.183	0.125	0.164
	3	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.004
Mdh	1	0.385	0.317	0.333	0.533	0.400	0.556	0.383	0.383	0.297	0.399
	2	0.615	0.650	0.667	0.433	0.600	0.444	0.617	0.617	0.703	0.594
	3	0.000	0.033	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.007
Pgm	1	0.500	0.583	0.600	0.433	0.600	0.639	0.550	0.617	0.641	0.574
	2	0.500	0.417	0.400	0.533	0.400	0.361	0.450	0.383	0.359	0.423
	3	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.004
Sdh	1	0.192	0.400	0.733	0.783	0.300	0.472	0.583	0.633	0.344	0.493
	2	0.808	0.600	0.267	0.217	0.700	0.528	0.417	0.367	0.656	0.507

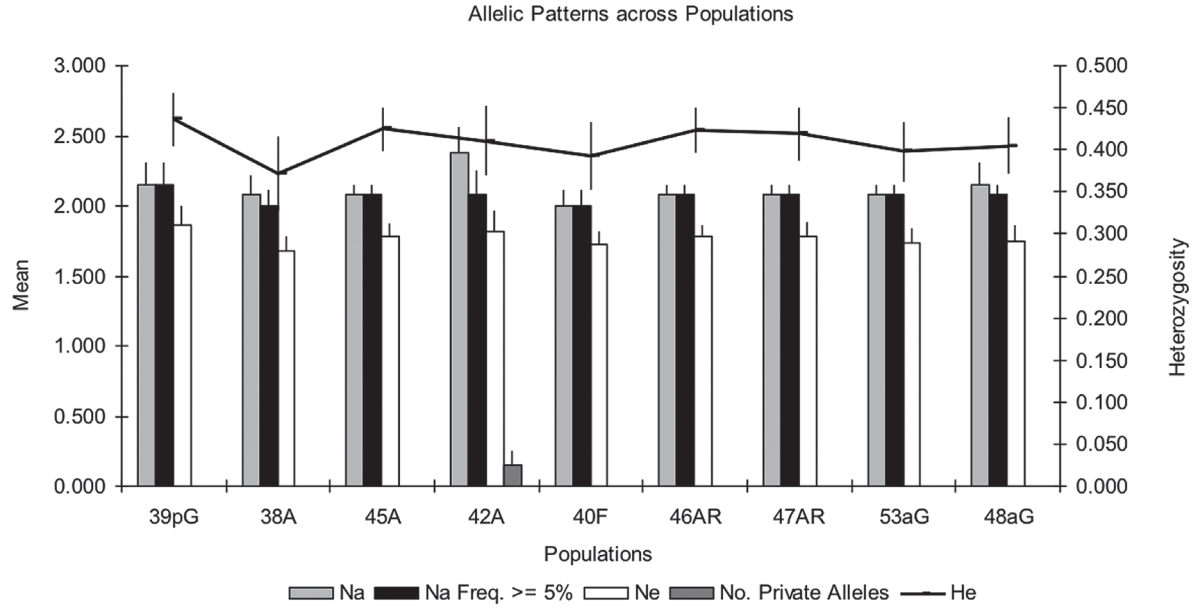

Fig. 5. Genetic variability parameters of *Anthoxanthum aristatum* populations
Explanations: N_a – number of alleles, N_e – number of effective alleles, H_e – expected heterozygosity; site numbers – see Appendix 1

est F = -0.203 in population (46fG), indicating excess of heterozygotes in these populations (Fig. 6, Table 7, Appendices 2-3).

Table 6. Private allele frequency in *Anthoxanthum aristatum* populations

Population	Locus	Allele	Freq.
42A	*Pgd*	3	0.033
42A	*Pgm*	3	0.033

Results of hierarchical AMOVA suggest that most of the observed variation is due to intrapopulation variation (85%), whereas interpopulation variation accounts for only 15% of the total variation (Table 8).

The first two principal components PC1 and PC2 jointly carry 43% of information about genetic similarity of populations of *A. aristatum* (Fig. 7). The analysis of principal components was based on Nei's genetic distances. Out of the group of individuals located in the central part of the diagram and composed of most

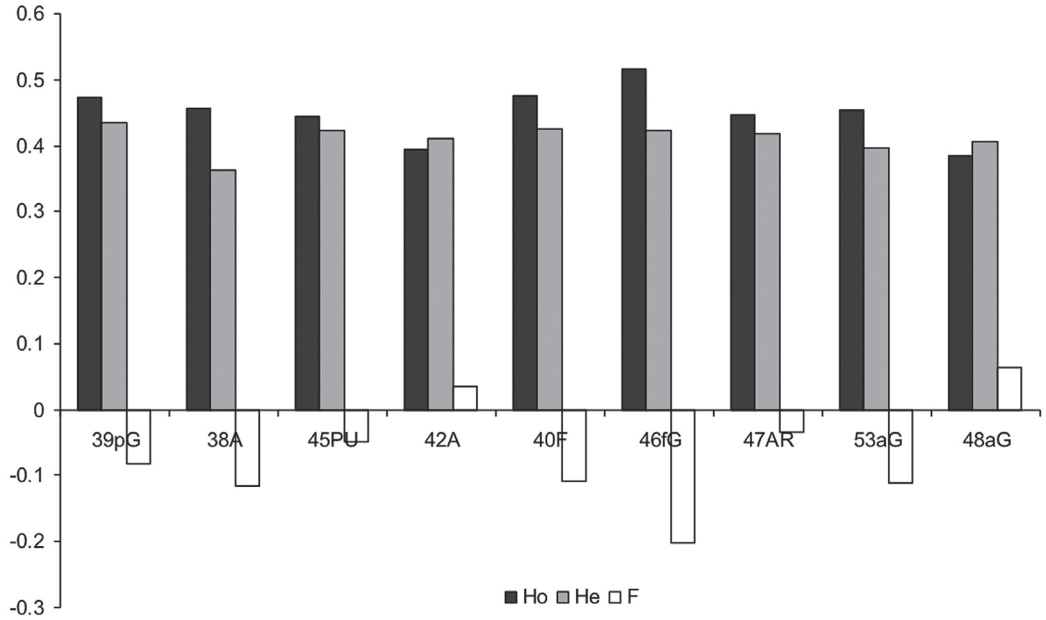

Fig. 6. Mean values of observed heterozygosity (H_o), expected heterozygosity (H_e) and fixation index (F) for *Anthoxanthum aristatum* populations for all loci

Table 7. Mean genetic diversity indices of *Anthoxanthum aristatum* populations

Population	*N*	*A*	N_e	*P* (%)	H_o	H_e	*F*
39pG	26	2.153	1.863	100.00	0.473	0.436	-0.081
38A	30	2.077	1.677	81.57	0.456	0.363	-0.116
45A	30	2.077	1.785	100.00	0.444	0.424	-0.049
42A	30	2.385	1.818	98.20	0.395	0.411	0.035
40F	10	2.000	1.721	91.67	0.475	0.426	-0.109*
46fG	18	2.077	1.780	100.00	0.517	0.423	-0.203*
47AR	30	2.077	1.786	90.00	0.446	0.418	-0.034
53aG	32	2.077	1.731	100.00	0.454	0.397	-0.112
48aG	30	2.154	1.752	100.00	0.385	0.405	0.064
Mean	26.2	2.135	1.768	98.33	0.449	0.411	-0.067

Explanations: *N* – sample size, *A* – number of alleles per locus, N_e – effective number of alleles, *P* – proportion of polymorphic loci, H_o – observed heterozygosity, H_e – expected heterozygosity, *F* – fixation index, * $p \leq 0.05$

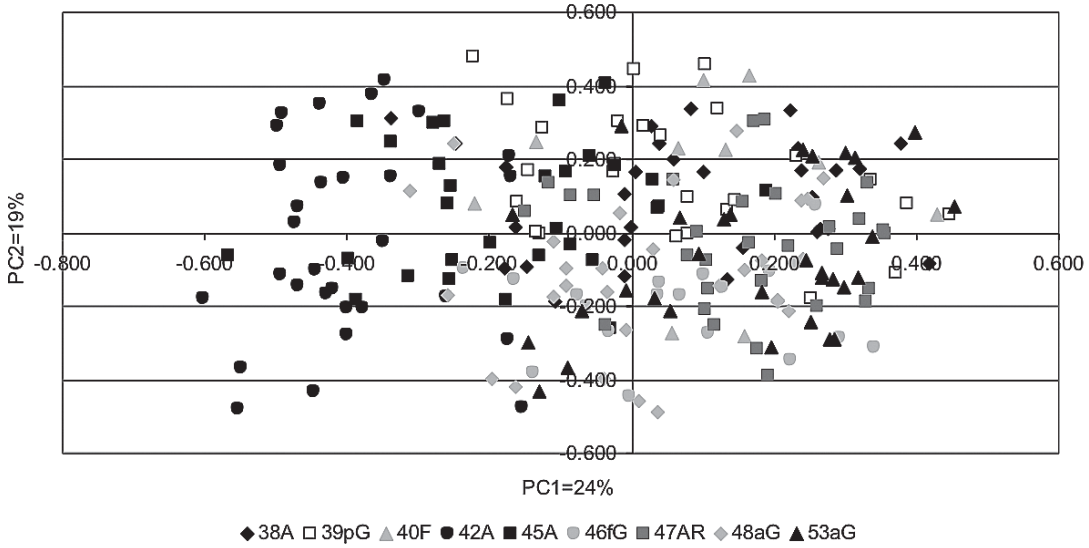

Fig. 7. Distribution of *Anthoxanthum aristatum* populations in the system of the first two principal components (PC1 and PC2), based on Nei's (1978) genetic distances
Explanations: site numbers – see Appendix 1

plants of the analysed populations, PCA distinguished a population from an arable field (42A) in the Noteć Forest and a population from an arable field near the nature reserve "Bagno Chlebowo" (45A), with negative values of PC1. The remaining populations form a loose group, with negative and positive values of PC1 and PC2. In the dendrogram (Fig. 8), two groups of similar populations are visible. The first one is composed of two populations: from an arable field (42A) and from fallow land (40F). The second group consists of a population

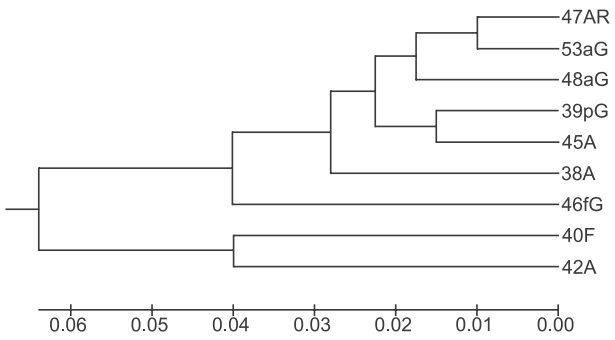

Fig. 8. UPGMA dendrogram for 9 *Anthoxanthum aristatum* populations, based on Nei's (1978) genetic distances
Explanations: site numbers – see Appendix 1

Table 8. Hierarchical molecular analysis of variance (AMOVA) for *Anthoxanthum aristatum* populations

Source of variation	Sum of squares	Component of variance	Variance (%)
Between populations	221.082	0.878	15
Within populations	1091.901	4.810	85
Total	1312.983	5.688	100

Table 9. Values of Wright' (1965) *F* statistics and gene flow at 13 loci of *Anthoxanthum aristatum* (Region 1 – Chlebowo, Region 2 – Nowy Tomyśl Sandur, Region 3 – Rzepin Forest), (see Appendix 1)

Locus	F_{IS}	F_{IT}	F_{ST}	N_m
Pgi-1	-0.293	-0.270	0.018	13.711
Pgi-2	-0.065	-0.010	0.052	4.558
Idh-1	-0.116	-0.069	0.043	5.627
Px-1	0.238	0.297	0.077	3.001
Px-2	0.094	0.206	0.123	1.780
Dia-1	-0.004	0.051	0.054	4.345
Dia-2	0.098	0.193	0.105	2.127
Got-1	-0.039	0.214	0.243	0.777
Got-2	0.042	0.240	0.207	0.956
Pgd	0.116	0.182	0.075	3.076
Mdh	-0.299	-0.259	0.031	7.796
Pgm	-0.430	-0.408	0.015	16.011
Sdh	-0.252	-0.069	0.146	1.460
Mean between populations	-0.055	0.031	0.087*	5.314
Mean between regions	-0.043	0.008	0.052*	4.579

Explanations: *A. aristatum* F_{IS} – inbreeding coefficient, F_{IT} – total inbreeding coefficient, F_{ST} – coefficient of genetic differentiation between populations, N_m – rate of gene flow between populations, $N_m = [(1/F_{ST}) - 1]/4$ (Pecal & Smose 2010 Genalex), * $p < 0.05$

Table 10. Ewens-Watterson neutrality test for all *Anthoxanthum. aristatum* populations

Locus	*N*	*A*	Obs. freq.	L95	U95
Pgi-1	472	2	0.659	0.503	0.996
Pgi-2	472	4	0.332	0.331	0.967
Idh	472	2	0.623	0.505	0.996
Px-1	472	2	0.503	0.504	0.996
Px-2	472	2	0.513	0.503	0.996
Dia-1	472	2	0.634	0.505	0.996
Dia-2	472	2	0.560	0.508	0.996
Got-1	472	2	0.544	0.504	0.996
Got-2	472	2	0.524	0.502	0.996
Pgd	472	3	0.729	0.400	0.992
Mdh	472	3	0.514	0.396	0.987
Pgm	472	3	0.506	0.385	0.987
Sdh	472	2	0.500	0.504	0.996

Explanations: *N* – sample size, *A* – number of alleles per locus, Obs. freq. – observed frequency of the locus, L95 and U95 – lower and upper 95% confidence limits of observed frequency

from grassland near pine forest (46fG), joined with the other 2 subgroups. The first one includes populations from arable fields (38A and 45A) and sandy grassland near pine forest plantation (39pG). The other subgroup is composed of plants from sandy grasslands near arable fields (48aG and 53aG), and a population from a field roadside (47AR).

Values of coefficients F_{IS}, F_{IT}, and F_{ST}, and N_m for all loci and their mean values are shown in Table 9. The F_{ST} value of 0.087 attests to low differentiation between populations, whereas gene flow between populations is relatively high ($N_m = 5.314$). The coefficient of genetic differentiation between regions ($F_{ST} = 0.052$) indicates a high similarity of populations from different parts of Poland, due to intensive gene flow between regions ($N_m = 4.579$).

Ewens-Watterson test (Manly 1985) for individual loci in each population as well as for all loci jointly shows that at 2 loci (*Px*-1 and *Sdh*), allele frequency is a result of selection. At the other loci, alleles were neutral (Table 10). A linkage disequilibrium test for the analysed loci detected linkage between loci *Pgi-2* and *Dia-1* in population 39pG.

3.3. Isozyme variation of *Anthoxanthum alpinum*

Genetic variation of populations of *A. alpinum* was analysed on the basis of 9 enzyme systems (Fig. 2, Table 1).

Phosphoglucoisomerase (PGI)

The gel zymograms were characteristic of dimeric enzymes. PGI activity was detected in 2 regions of the anodal part of the gel. Locus *Pgi*-1 was composed of 2 alleles. Homozygotes had a single band, while heterozygotes had 3 bands. Locus *Pgi*-2 was monomorphic.

Isocitrate dehydrogenase (IDH)

The gel zymograms were characteristic of a dimeric form of the enzyme. Activity was detected in the anodal part of the gel at one locus with 2 alleles.

Peroxidase (PX)

PX was encoded at 2 loci: *Px*-1 in the anodal part of the gel, with 3 alleles, whereas *Px*-2 was monomorphic. Phenotypes were characteristic of dimeric enzymes: homozygotes with a single band and heterozygotes with 3 bands.

NADH diaphorase (DIA)

Activity of monomeric DIA was recorded in 2 regions of the anodal part of the gel. Locus *Dia*-1 was composed of 2 alleles, whereas locus *Dia*-2, of 4 alleles. Homozygotes had a single band, while heterozygotes had 2 bands.

Glutamate oxaloacetic transaminase (GOT)

The gel zymograms were characteristic of dimeric enzymes. Activity of the enzyme was detected in 2 regions of the anodal part of the gel and labelled as *Got*-1 and *Got*-2. At each of them, 2 alleles were found. One-band phenotypes were detected in homozygotes and 3-band phenotypes in heterozygotes.

Phosphogluconate dehydrogenase (PGD)

Dimeric PGD was detected in the anodal part of the gel at a single locus *Pgd-1*, with 2 alleles.

Malate dehydrogenase NAD^+(MDH)

Activity of dimeric MDH was detected at a single locus *Mdh*-1 with 4 alleles, in the anodal part of the gel. Phenotypes were composed of 1 or 3 bands.

Phosphoglucomutase (PGM)

Activity of monomeric PGM was detected in the anodal part of the gel at a single locus, with 3 alleles.

Shikimate dehydrogenase (SHD)

The enzyme was detected in the anodal part of the gel at a single locus *Shd*-1, with 2 alleles. The phenotype was characteristic of monomeric enzymes.

Allele frequencies varied within populations and between populations. The most frequent were: allele 1 at locus *Idh*, allele 1 at *Dia*-1, allele 1 at locus *Got*-1 and allele 2 at locus *Got*-2 (Table 11).

Genetic variability parameters of *A. alpinum* populations (Tables 11-13, Appendix 2) indicate that loci *Pgi*-2 and *Px*-2 were monomorphic in all the populations. Additionally, some loci were monomorphic in single populations: *Got*-1 in population 56SM, while *Dia*-1, *Got*-1, and *Got*-2 in population 57SG. In alpine population 55AG (Diablak, i.e. main peak of the Babia Góra massif), H_o and H_e did not differ markedly at individual loci, whereas inbreeding coefficients (F) were negative at 6 loci: *Dia*-1, *Dia*-2, *Got*-1, *Got*-2, *Mdh*, and *Pgm*. Its values were positive at locus *Pgi*-1, *Idh, Pgd*, *Sdh* (Fig. 9, Table 12). In subalpine population 56SM, a slight deviation from Hardy-Weinberg equilibrium was found at loci *Dia*-2, *Pgm*, and *Sdh*. In subalpine population 57SG, values of H_o and H_e are very similar to each other. Inbreeding coefficient had negative values at loci *Pgi*-1, *Idh*, *Dia*-2, *Mdh*, *Pm*, and *Sdh*, whereas a small excess of homozygotes was noted at loci *Px*-1 and *Pgd*. In upper montane population 58UG (Markowe Szczawiny), small differences were found between H_o and H_e at most loci, and F had negative values except for locus *Pgd*. For all loci F coefficient had negative values in populations 56SM, 57SG and 58UG, but values close to Hardy-Weinberg equilibrium were recorded in population 55AG (Fig. 10).

The most polymorphic populations were 55MW from Diablak and 58PG from Markowe Szczawiny, while subalpine population 57PS near a trail was the least polymorphic (Table 13).

Genetic differentiation between populations was relatively high (F_{ST}=0.198), whereas gene flow between populations was low (N_m = 1.709) (Table 14). Ewens-Watterson test (Manly 1985) for individual loci in each population as well as for all loci jointly showed that all loci were neutral (Table 15). No linkage was detected between loci in populations of *A. alpinum*. Populations 56SM and 58UG are distinguished by the presence of private alleles at 2 loci: *Mdh* and *Pgm* (Table 16). Results of hierarchical AMOVA suggest that most of the observed variation is due to intrapopulation variation (83%) (Table 17). Grouping by UPGMA, based on Nei's genetic distances, shows similarity between populations from subalpine and alpine grasslands (55AG, 56SM, 57SG), whereas the upper montane population (58UG) clearly differs from the others (Fig. 11, Table 18).

The first two principal components, calculated on the basis of Nei's genetic distances (1978) jointly carry 93% of information about the observed variation. In the diagram, none of the studied populations is clearly distinct from the others. Only PC1 differentiates individuals from upper montane population 58UG: 3 of them have negative values and the others have positive values of PC1 (Fig. 12).

Table 11. Allele frequency in *Anthoxanthum alpinum* populations

Locus	Allele	55AG	56SM	57SG	58UG
Pgi-1	1	0.735	0.714	0.688	0.500
	2	0.265	0.286	0.313	0.500
Pgi-2	2	1.000	1.000	1.000	1.000
Idh	1	0.824	0.786	0.875	0.417
	2	0.176	0.214	0.125	0.583
Px-1	1	0.029	0.036	0.000	0.417
	2	0.647	0.714	0.250	0.583
	3	0.324	0.250	0.750	0.000
Px-2	1	1.000	1.000	1.000	1.000
Dia-1	1	0.912	0.929	1.000	0.833
	2	0.088	0.071	0.000	0.167
Dia-2	1	0.000	0.000	0.188	0.917
	2	0.000	0.179	0.188	0.083
	3	0.471	0.357	0.438	0.000
	4	0.529	0.464	0.188	0.000
Got-1	1	0.941	1.000	1.000	0.750
	2	0.059	0.000	0.000	0.250
Got-2	1	0.088	0.107	0.000	0.583
	2	0.912	0.893	1.000	0.417
Pgd	1	0.529	0.321	0.250	0.750
	2	0.471	0.679	0.750	0.250
Mdh	1	0.735	0.571	0.000	0.417
	2	0.088	0.250	0.750	0.167
	3	0.176	0.107	0.250	0.417
	4	0.000	0.071	0.000	0.000
Pgm	1	0.294	0.571	0.250	0.000
	2	0.706	0.429	0.750	0.583
	3	0.000	0.000	0.000	0.417
Sdh	1	0.206	0.464	0.750	0.167
	2	0.794	0.536	0.250	0.833

Explanations: 55AG – Diablak, alpine grassland, 56SM – Przełęcz Brona, subalpine matgrass meadow, 57SG – subalpine grassland near trail, 58UG – Markowe Szczawiny, upper montane forest glade

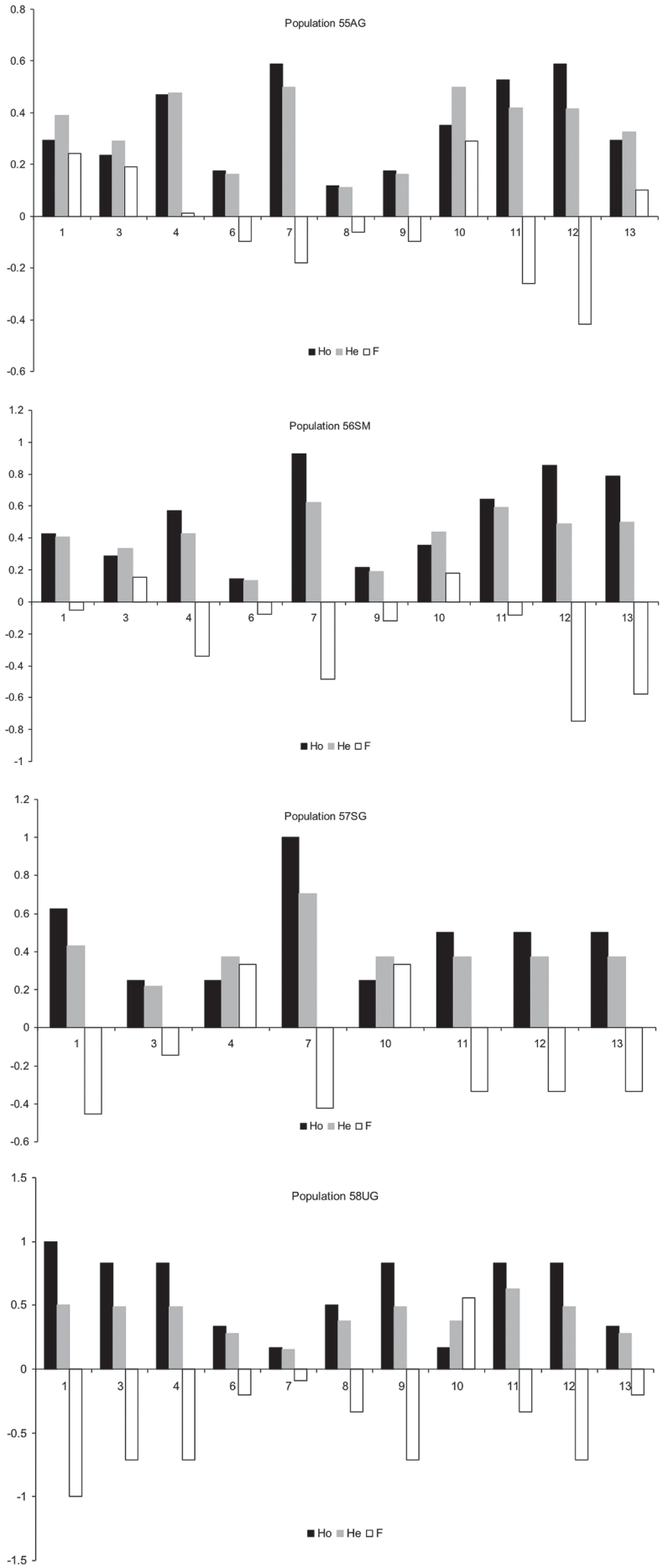

Fig. 9. Mean values of observed heterozygosity (*Ho*), expected heterozygosity (*He*) and fixation index (*F*) in *Anthoxanthum alpinum* populations for loci 1-13 (see Table 11)

Table 12. Genetic diversity indices of *Anthoxanthum alpinum* populations

Population	Locus	N	A	N_e	H_o	H_e	F
55AG	*Pgi*-1	17	2	1.637	0.294	0.389	0.244
	Pgi-2	17	1	1.000	-	-	-
	Idh	17	2	1.410	0.235	0.291	0.190
	Px-1	17	3	1.908	0.471	0.476	0.011
	Px-2	17	1	1.000	-	-	-
	Dia-1	17	2	1.192	0.176	0.161	-0.097
	Dia-2	17	2	1.993	0.588	0.498	-0.181
	Got-1	17	2	1.125	0.118	0.111	-0.063
	Got-2	17	2	1.192	0.176	0.161	-0.097
	Pgd	17	2	1.993	0.353	0.498	0.292
	Mdh	17	3	1.725	0.529	0.420	-0.259
	Pgm	17	2	1.710	0.588	0.415	-0.417
	Sdh	17	2	1.486	0.294	0.327	0.101
56SM	*Pgi*-1	14	2	1.690	0.429	0.408	-0.050
	Pgi-2	14	1	1.000	-	-	-
	Idh	14	2	1.508	0.286	0.337	0.152
	Px-1	14	3	1.742	0.571	0.426	-0.341
	Px-2	14	1	1.000	-	-	-
	Dia-1	14	2	1.153	0.143	0.133	-0.077
	Dia-2	14	3	2.667	0.929	0.625	-0.486
	Got-1	14	1	1.000	-	-	-
	Got-2	14	2	1.237	0.214	0.191	-0.120
	Pgd	14	2	1.774	0.357	0.436	0.181
	Mdh	14	4	2.465	0.643	0.594	-0.082
	Pgm	14	2	1.960	0.857	0.490	-0.750
	Sdh	14	2	1.990	0.786	0.497	-0.579
57SG	*Pgi*-1	8	2	1.753	0.625	0.430	-0.455
	Pgi-2	8	1	1.000	-	-	-
	Idh	8	2	1.280	0.250	0.219	-0.143
	Px-1	8	2	1.600	0.250	0.375	0.333
	Px-2	8	1	1.000	-	-	-
	Dia-1	8	1	1.000	-	-	-
	Dia-2	8	4	3.368	1.000	0.703	-0.422
	Got-1	8	1	1.000	-	-	-
	Got-2	8	1	1.000	-	-	-
	Pgd	8	2	1.600	0.250	0.375	0.333
	Mdh	8	2	1.600	0.500	0.375	-0.333
	Pgm	8	2	1.600	0.500	0.375	-0.333
	Sdh	8	2	1.600	0.500	0.375	-0.333
58UG	*Pgi*-1	6	2	2.000	1.000	0.500	-1.000
	Pgi-2	6	1	1.000	-	-	-
	Idh	6	2	1.946	0.833	0.486	-0.714
	Px-1	6	2	1.946	0.833	0.486	-0.714
	Px-2	6	1	1.000	-	-	-
	Dia-1	6	2	1.385	0.333	0.278	-0.200
	Dia-2	6	2	1.180	0.167	0.153	-0.091
	Got-1	6	2	1.600	0.500	0.375	-0.333
	Got-2	6	2	1.946	0.833	0.486	-0.714
	Pgd	6	2	1.600	0.167	0.375	0.556
	Mdh	6	3	2.667	0.833	0.625	-0.333
	Pgm	6	2	1.946	0.833	0.486	-0.714
	Sdh	6	2	1.385	0.333	0.278	-0.200

Explanations: N – sample size, A – number of alleles per locus, N_e – number of effective alleles, H_o – observed heterozygosity, H_e – expected heterozygosity, F – fixation index, 55AG – Diablak, alpine grassland, 56SM – Przełęcz Brona, subalpine matgrass meadow, 57SG – subalpine grassland near trail, 58UG – Markowe Szczawiny, upper montane forest glade

Table 13. Mean values of genetic diversity indices in *Anthoxanthum alpinum* populations

Population	*N*	*A*	N_e	*P* (%)	H_o	H_e	*F*
55AG	17	1.900	1.490	84.6	0.294	0.288	-0.025
56SM	14	2.077	1.630	76.9	0.401	0.318	-0.215*
57SG	8	1.769	1.492	61.5	0.298	0.248	-0.169*
58UG	6	1.923	1.662	84.6	0.513	0.348	-0.405*
Mean	-	1.942	1.568	76.9	0.376	0.301	-0.204*

Explanations: *N* – sample size, *A* – number of alleles per locus, N_e – number of effective alleles, *P* – proportion of polymorphic loci, H_o – observed heterozygosity, H_e – expected heterozygosity, *F* – fixation index, 55AG – Diablak, alpine grassland, 56SM – Przełęcz Brona, subalpine matgrass meadow, 57SG – subalpine grassland near trail, 58UG – Markowe Szczawiny, upper montane forest glade,* $p \leq 0.05$

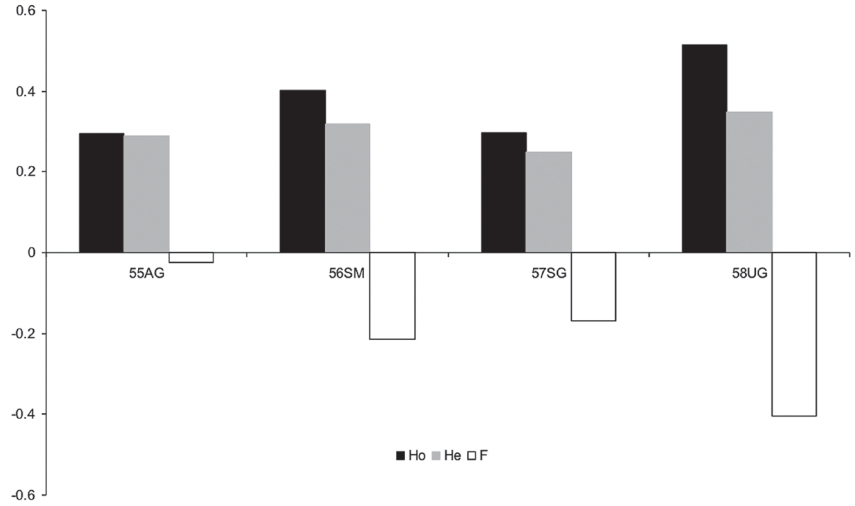

Fig. 10. Mean values observed heterozygosity (*Ho*), expected heterozygosity (*He*) and fixation index (*F*) in *Anthoxanthum alpinum* populations for all loci

Table 14. Values of Wright' (1965) *F* statistics and gene flow for all loci in *Anthoxanthum alpinum* populations

Locus	F_{IS}	F_{IT}	F_{ST}	N_m
Pgi-1	-0.359	-0.306	0.039	6.174
Pgi-2	-0.600	-0.103	0.310	0.556
Idh	-0.157	0.067	0.194	1.040
Px-1	-0.170	0.098	0.229	0.843
Px-2	0.467	0.632	0.310	0.556
Dia-1	-0.226	-0.114	0.091	2.500
Dia-2	-0.353	0.049	0.297	0.592
Got-1	-0.116	-0.048	0.061	3.872
Got-2	-0.390	0.125	0.371	0.425
Pgd	0.320	0.383	0.094	2.414
Mdh	-0.184	0.082	0.224	0.865
Pgm	-0.541	-0.296	0.159	1.322
Sdh	-0.321	-0.069	0.191	1.060
Mean	-0.202	0.038	0.198	1.709

Explanations: *A. aristatum* F_{IS} – inbreeding coefficient, F_{IT} – total inbreeding coefficient, F_{ST} – coefficient of genetic differentiation between populations, N_m – rate of gene flow between populations, $N_m = [(1/F_{ST}) - 1]/4$ (Pecal & Smose 2010, Genalex)

Table 15. Ewens-Watterson neutrality test for all *Anthoxanthum alpinum* populations

Locus	*N*	*A*	Obs. freq.	L95	U95
Pgi-1	90	2	0.571	0.502	0.98
Pgi-2	90	1	1.000	-	-
Idh	90	2	0.631	0.502	0.98
Px-1	90	3	0.464	0.376	0.96
Px-2	90	1	1.000	-	-
Dia-1	90	2	0.857	0.502	0.98
Dia-2	90	4	0.311	0.309	0.89
Got-1	90	2	0.895	0.502	0.98
Got-2	90	2	0.753	0.501	0.98
Pgd	90	2	0.506	0.504	0.98
Mdh	90	4	0.373	0.307	0.89
Pgm	90	3	0.488	0.368	0.96
Sdh	90	2	0.530	0.502	0.98

Explanations: *N* – sample size, *A* – number of alleles per locus, Obs. freq. – observed frequency of the locus, L95 and U95 – lower and upper 95% confidence limits of observed frequency

Table 16. Private allele frequency in *Anthoxanthum alpinum* populations

Population	Locus	Allele	Freq.
56AG	*Mdh*	4	0.071
58UG	*Pgm*	3	0.417

Table 17. Analysis of molecular variance (AMOVA) for *Anthoxanthum alpinum* populations

Source of variation	Sum of squares	Mean square	Component of variance	Variance (%)
Among populations	32.842	10.947	0.418	17
Within populations	174.614	2.030	2.030	83
Total	207.456	12.978	2.448	100

Table 18. Nei's (1978) genetic similarity between *Anthoxanthum alpinum* populations

	55AG	56SM	57SG	58UG
56SM	0.028			
57SG	0.129	0.090		
58UG	0.196	0.231	0.314	

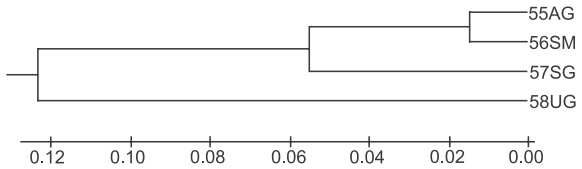

Fig. 11. UPGMA dendrogram of *Anthoxanthum alpinum* populations, based on Nei's (1978) genetic distances
Explanations: site numbers – see Appendix 1

3.4. Morphological versus isozyme variation of *Anthoxanthum odoratum*, *A. alpinum*, and *A. aristatum*

3.4.1. Morphological versus isozyme variation of *Anthoxanthum odoratum*

In the populations of *A. odoratum* analysed in respect of isozyme variation, also morphological variation was studied (Drapikowska 2013). On the basis of discriminant analysis (Fig. 13), the most distinct populations are in a lower montane meadow (29LM), pine forest plantation (19PP), and sandy grassland near the pine forest plantation (18pG). The analysis of isozyme variation also distinguished populations 19PP and 29LM (Fig. 3). The other populations were characterized by differences between patterns of morphological and isozyme variation.

3.4.2. Morphological versus isozyme variation of *Anthoxanthum aristatum*

Coefficients of Spearman rank correlation (Lange 1995) between Nei's genetic distances and Mahalanobis distances based on morphological data, calculated for pairs of populations of *A. aristatum*, were not statistically significant (r = 0.234, Fig. 14). Distribution of populations in the system of the first two canonical variables, based on morphological characters, shows the distinctness of populations in a sandy grassland near a pine forest plantation (39pG) and in an arable field (42A, Fig. 15). The pattern of genetic variation based on Nei's genetic distances shows the distinctness of population 42A and 40F. The other populations had different patterns of morphological and isozyme variation (Fig. 7-8).

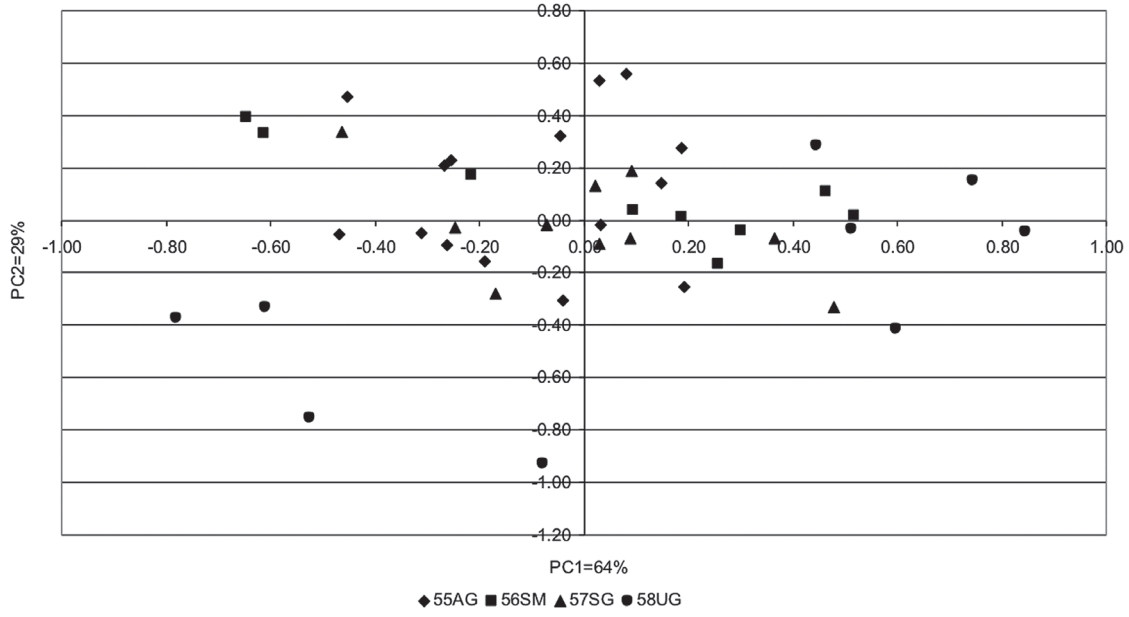

Fig. 12. Distribution of *Anthoxanthum alpinum* populations in the system of the first two principal components (PC1 and PC2), based on Nei's (1978) genetic distances
Explanations: site numbers – see Appendix 1

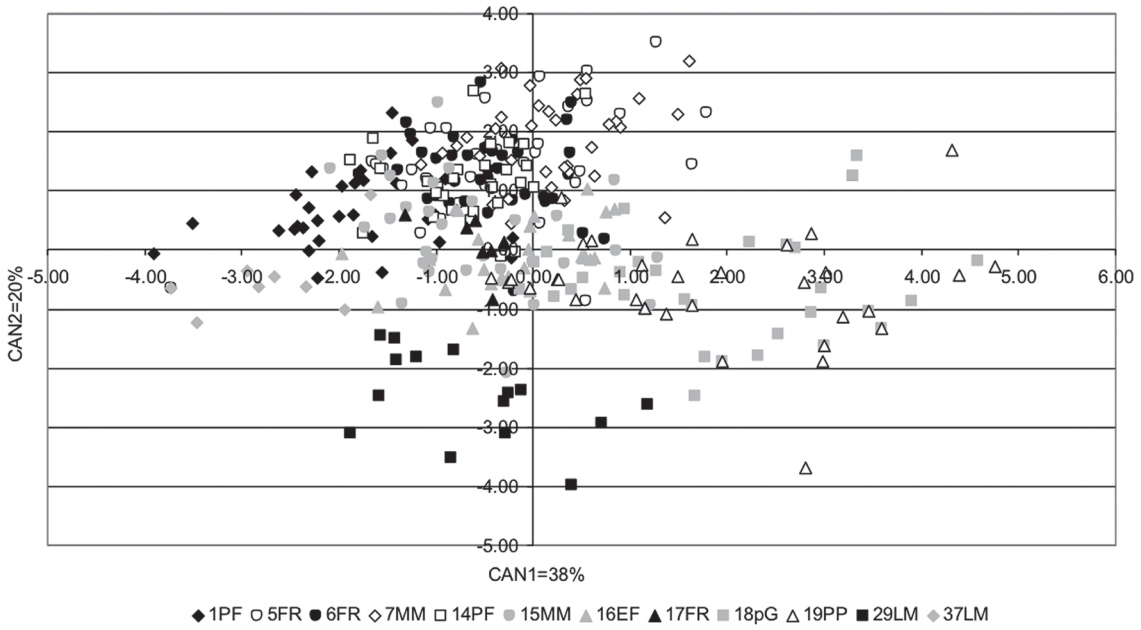

Fig. 13. Distribution diagram of *Anthoxanthum odoratum* specimens from various habitats in the system of the first two canonical variables (CAN1 and CAN2)
Explanations: site numbers – see Appendix 1

3.4.3. Morphological versus isozyme variation of *Anthoxanthum alpinum* along the altitudinal transect

To determine if the observed morphological variation of populations of *A. alpinum* is correlated with detected isozyme variation of the same populations, Coefficient of Spearman rank correlation between Nei's genetic distances and Mahalanobis distances based on morphological data was calculated. The correlation is positive ($r = 0.54$), significant at $p < 0.05$ (Fig. 16).

Distribution of populations in the system of the first two canonical variables, based on morphological characters, shows the distinctness of the population from a subalpine grassland near a trail (57SG) (Drapikowska 2013). The pattern of genetic variation based on Nei's genetic distances differs from the pattern of morphological

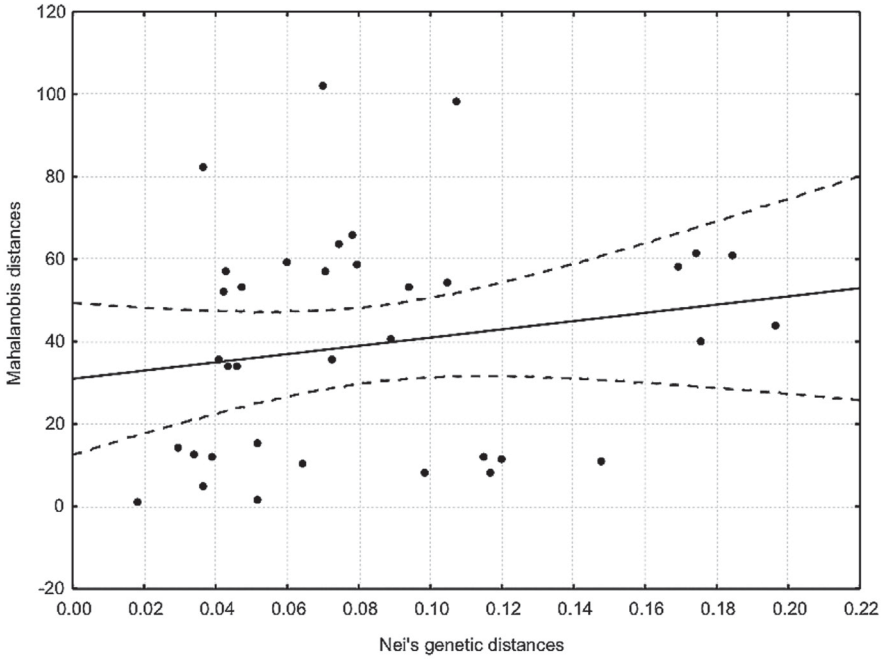

Fig. 14. Spearman rank correlation between Nei's (1978) genetic distances and Mahalanobis distances for *Anthoxanthum aristatum* populations

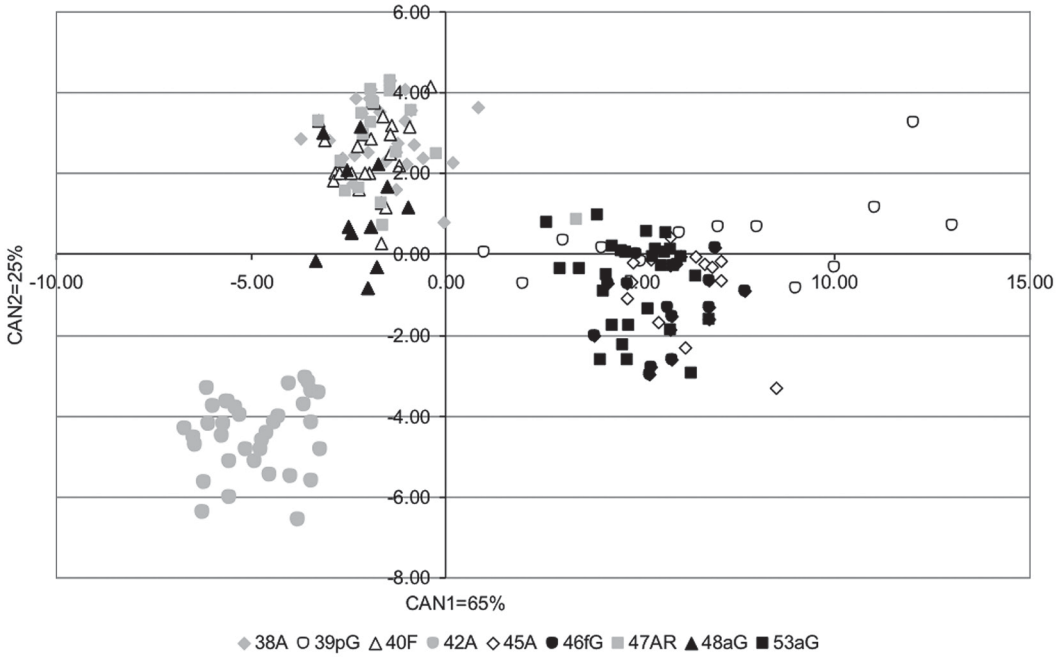

Fig. 15. Distribution diagram of *Anthoxanthum aristatum* specimens from various habitats in the system of the first two canonical variables (CAN1 and CAN2)
Explanations: site numbers – see Appendix 1

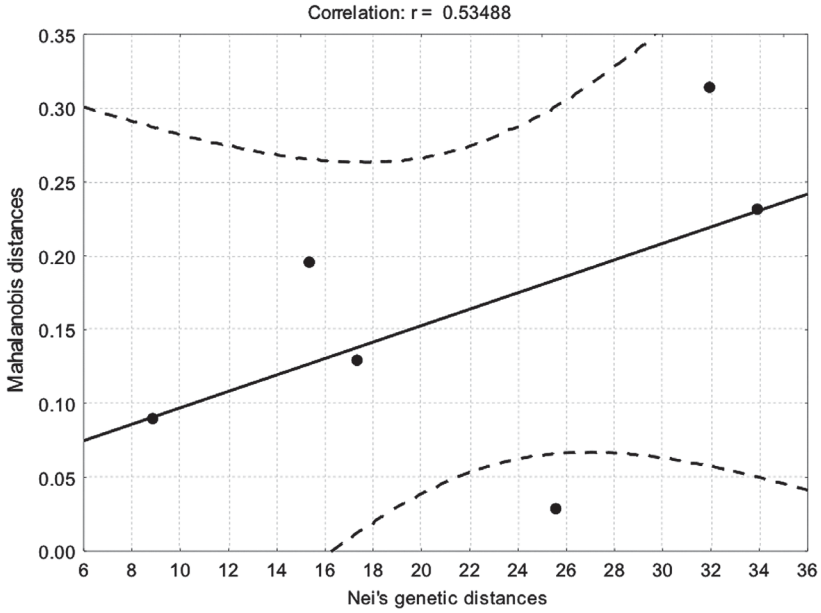

Fig. 16. Correlations between Nei's (1978) genetic distances and Mahalanobis distances for *Anthoxanthum alpinum* populations

variation. The analysis shows the distinctness of the upper montane population (58UG) (Fig. 11).

4. Discussion

4.1. Variation of *Anthoxanthum odoratum*

Anthoxanthum odoratum within its natural geographical range is highly variable phenotypically and genetically. Interpopulation variation is observed in both West Europe (Pimentel & Sahuquillo 2008) and Central Europe, e.g. in Poland (Drapikowska *et al*. 2011; Drapikowska 2013).

Habitat type only slightly affects the pattern of variation within *A. odoratum* (see Fig. 3). The observed morphological variation is poorly correlated with habitat type and variation in soil conditions (Drapikowska 2013). Similarly, the observed isozyme variation is

only slightly conditioned by environmental pressure. The diversifying effects of drift and selection may have been diminished by long-distance gene flow, mediated by wind-pollination (Dixon 2002). Similar conclusions were earlier drawn on the basis of experiments in controlled conditions (Silvertown *et al.* 2006; Freeland *et al.* 2010, 2012). They show that selection pressure, caused by different environmental conditions, affects interpopulation variation.

This study shows the distinctness of montane populations, which is probably associated with geographical isolation. In lowland populations, occupying many types of habitats, there is a high level of isozyme variation. *A. odoratum*, spreading to new sites linked with human interference – roadsides, edges of pine forest plantations – crosses successive ecological barriers and is subject to the adaptation process (Antonovics 1972).

Ecological expansion of *A. odoratum* is possible thanks to its ability to tolerate a wide range of environmental changes. Phenotypic plasticity is characteristic of polyploids (Mizianty 1994), such as *Phragmites australis* (Drapikowska & Krzakowa 2009) and *Calamagrostis arundinacea* (Krzakowa & Celka 2007, 2008). Besides, the lack of barriers that could block gene flow between populations of *A. odoratum*, which is highly allogamous and reproduces also sexually, contributes to diminishing of differences between populations occupying various habitats. However, little support was found for a consistent relationship between isozyme variation and morphological variation. Similar findings were obtained in a study of *Briza media* (Ellmer *et al.* 2011).

Anthoxanthum odoratum is an allopolyploid, deriving from diploid ancestors whose genomes were similar to those of present-day *A. ovatum* and *A. alpinum* (Borrill 1963; Jones 1964). Cytological research aimed to assess the number of rDNA loci and DNA content in *A. odoratum*, has revealed complex rearrangements within the *Anthoxanthum* genome, consisting in deletion and insertion of DNA segments (Drapikowska *et al*. 2013). Isozyme analyses made by Zeroual-Humbert-Droz & Felber (1999) suggested an autopolyploid origin of *A. odoratum*, but the present study, using isozyme markers, confirms its allopolyploid origin (see Fig. 2).

4.2. Variation of *Anthoxanthum aristatum*

Within its primary distribution range, *A. aristatum* is highly variable both genetically and morphologically (Pimentel *et. al* 2007, 2010). This species is invasive in Poland (Latowski 2005; Tokarska-Guzik 2005). It is assumed that invasive species are characterized by high genetic variability, higher in invaded areas than in populations from the natural range (Lavergne & Molofsky 2007). This is associated with the need for defence and competitiveness. Many models have been developed to describe the causes of success of invasive plants (e.g. Barrett & Shore 1989; Blossey & Nötzold 1995). The evolution of increased competitive ability (EICA) model suggests that the competitiveness of invasive species is stimulated by contact with native plants. The mating system is one of the major factors explaining genetic variation among populations of one species. Based on isozyme loci (Hamrick & Godt 1996), genetic differentiation of *A. aristatum* fits within the range typical of other wind-pollinated species with cross-pollination (F_{ST} = 0.1, Hamrick & Loveless 1986).

Genetic differentiation between populations is low (F_{ST} = 0.087), and between regions it is even lower (F_{ST} = 0.052). This result is comparable to that reported by Krzakowa and Dunajski (2007) for populations of *Calamagrostis arundinacea*, where F_{ST} = 0.0565. The low differentiation is mostly due to intensive gene flow and allogamy of this species. Genetic and morphological analyses of the grass *Phalaris arundinacea*, invasive in North America (Gifford *et al.* 2002), also showed a low morphological variation between populations from various habitats. Populations of *A. aristatum* are highly polymorphic (*P* = 98.33%) but variation within populations accounts for as much as 85% of the total variation. The high proportion of polymorphic loci in its populations of *A. aristatum* indicates that it has a high potential for adaptation. Most of the populations are in Hardy-Weinberg equilibrium, except 46fG and 40F, where an excess of heterozygotes was found. This may result from natural selection, which favours heterozygosity (Mitton 1989). However, Ewens-Watterson test shows that only 2 loci (*Px*-1 and *Sdh*) are subject to selection, whereas the other loci are neutral. Among all populations, only population 42A is distinct to some extent, thanks to private alleles at 2 loci: *Pgm* and *Pgm*. This species, spreading outside natural ecosystems (within the primary distribution range), shows an ability to invade secondary habitats of various types, markedly deviating from those occupied by the species originally. This results from the high polymorphism within populations of this species in the present study. It attests to a high plasticity of the species, which may favour its expansion to new anthropogenic sites, i.e. more fertile arable fields (Kapeluszny & Haliniarz 2010). The process of expansion of *A. aristatum* and colonization of new ecological niches has been observed in Poland for only several decades (Szmeja 1996; Skrajna & Skrzypczyńska 2007), so it cannot be expected that microevolutionary processes within such a short time would allow selection of stable genotypes characteristic of different ecological niches. Results of this study indicate a high viability of *A. aristatum* populations, irrespective of habitat type. This is related to colonization of new, more fertile sites by this species and its

expansion towards Eastern Europe.

Interspecific hybridization leads to creation of new genotypes and, consequently, to an increased viability of invasive species (Ellstrand & Schierenbeck 2000; Prentis *et al.* 2008). Within the primary distribution range populations of *A. aristatum* are sympatric with populations of the closely related *A. ovatum*, associated with pastures and open forests of the coasts and mountains of Tunisia and Morocco (Djebaili 1990; Pimentel *et al.* 2010). This sometimes leads to introgression between them (Jones 1964). Populations of *A. aristatum* and *A. odoratum* in Poland are sometimes sympatric, too, but no potential interspecific hybrids have been found. This is confirmed by morphological investigations (Drapikowska 2013) and the present study of isozymes. Crossing of these 2 taxa is difficult because of differences in their ploidy (Borrill 1963). Moreover, a comparative cytogenetic study has detected differences in genome size and number of rDNA loci between the 2 species, indicating substantial rearrangements within their genomes (Drapikowska *et al.* 2013).

Results of the present isoenzymatic study indicate that some groups of *A. aristatum* populations can be distinguished, but they are not always correlated with habitat type or geographical location.

4.3. Variation of *Anthoxanthum alpinum* versus *A. odoratum*

Genetic and morphological differentiation along the altitudinal transect has been investigated in many plant species, including grasses, e.g. *Briza media* (Hahn *et al.* 2012) and *Dactylis glomerata* (Lumaret 1984). Also genome size has been analysed in *Dactylis glomerata* in relation to altitude (Reeves *et al.* 1998). For many years, effects of altitude on morphological, genetic, and cytological variation of *A. odoratum* and *A. alpinum* have been studied in the Western Alps, Massif Central in France, and the Karkonosze Mts. in Poland and Czech Republic (Felber 1988; Bretagnolle 2001; Filipová & Krahulec 2006).

This study shows genetic variation of *A. alpinum* populations from the Babia Góra massif along the altitudinal transect. The mean percentage of polymorphic loci (P = 76.925%) is higher than that reported by Zhao Gui-Fang *et al.* (2001) for *A. alpinum* populations from the Swiss Alps (P = 64%) and by Zeroual-Humbert-Droz and Felber (1999) for populations from the French Alps. For allogamous plants, the mean percentage of polymorphic loci is P = 51.0% (Hamrick & Godt 1989). The mean expected heterozygosity H_e = 0.301 in the present study is higher than H_e = 0.252, reported by Zhao Gui-Fang *et al.* (2001). Among the analysed populations, the most polymorphic were those from Diablak (55AG) and Markowe Szczawiny (58UG), whereas the least polymorphic sample was that collected near a trail on Przełęcz Brona (56SM). Private alleles were found in populations 56SM (at locus *Mdh*) and 58UG (at locus *Pgm*). In comparison with populations from the Alps, much higher values of genetic differentiation were recorded in this study: F_{ST} = 0.061 for *Got*-1, F_{ST} = 0.371 for *Got*-2, F_{ST} = 0.229 for *Px*-1, F_{ST} = 0.310 for *Px*-2, and F_{ST} = 0.094 for *Pgd*-1 (Table 14). Mean genetic differentiation among populations was relatively high (F_{ST} = 0.198), and gene flow was relatively low (N_m = 1.709). In the Swiss Alps, total genetic variation was low, but remarkable differences were found between individual subpopulations (Zhao *et al.* 2001). Genetic variation of vascular plants along altitudinal transects shows various patterns of variability. In some species, genetic variation increases with altitude, while in others it decreases with increasing altitude (Ohsawa & Ide 2008) or variation at the highest and lowest altitudes is higher than in the middle part of the altitudinal range. Clinal variation of isozyme loci is observed along the altitudinal gradient of diploid and tetraploid populations of *Dactylis glomerata* (Lumaret 1984). Change in allele frequencies, correlated with altitude, was reported by Zhao *et al.* (2001). By contrast, allele frequencies in populations from Babia Góra are not correlated with altitude. Nei's (1978) genetic distances between subalpine and alpine populations were small (0.028), and a similar value was recorded for alpine populations in Arpette (2780 m) (Zhao *et al.* 2001). Nei's (1978) genetic distance between the alpine population and the population from Markowe Szczawiny (located about 530 m lower), amounts to 0.12. A similar value has been reported for populations in the Swiss Alps, located at very different altitudes, about 1000 m a.s.l away from each other (Zhao *et al.* 2001). *A. alpinum* on Babia Góra has partly isolated local populations. The largest local populations were found in the alpine and subalpine zone, whereas the population in the upper montane zone is currently represented by 6 individuals, i.e. 13 less than 3 years earlier. Thus the observed distinctness of this population may be partly due to the small sample size as well as its isolation from alpine and subalpine populations. The pattern of variation of *A. alpinum* in the Babia Góra massif is partly shaped by human activity and partly by genetic drift, which also conditions the variation of many alpine species (Stoöcklin *et al.* 2009).

An analysis of relations between the tetraploid *A. odoratum* and the diploid *A. alpinum* has shown that the contact zone between *A. alpinum* and *A. odoratum* is very narrow (Mirek & Piękoś-Mirkowa 2003). Populations of *A. odoratum* are found at lower altitudes, to the upper montane zone, where they are replaced by *A. alpinum*, whose altitudinal range reaches up to the alpine zone. Considering that *Anthoxanthum* species are cross-pollinated (like a majority of grasses), the 2

taxa potentially may hybridize. Bretagnolle (2001) suggests that triploid interspecific hybrids between them are possible. In the Babia Góra massif, the contact zone is very narrow, and species composition of vegetation in this zone is variable, mostly due to human activity (tourism, modification of tourist trails, felling of trees). The analysis of isozyme markers and flow cytometry show that the upper montane population is currently composed of individuals identified as *A. alpinum*.

5. Conclusions

- The observed isozyme variation of lowland populations of *Anthoxanthum odoratum* is poorly correlated with habitat type and the phase of ecological expansion.
- Populations of *A. odoratum* in the lower montane zone are characterized by partial genetic distinctness, reflected in frequency of bands of all the analysed isozyme loci.
- Five *Pgm* isozyme markers (*Pgi*-2, *Dia-2*, *Mdh*, *Idh, Pgm*) characteristic of the polyploid *A. odoratum* have been identified.
- *A. aristatum* is characterized by high intrapopulation polymorphism, low interpopulation variation, and intensive gene flow between populations.
- Variation of *A. aristatum* is poorly correlated with habitat type.
- *A. alpinum* shows genetic differentiation of populations along the altitudinal transect. Alpine and subalpine populations are distinct from the upper montane population. This is reflected in lower values of inbreeding coefficient (*F*) and Nei's genetic distance.
- Populations of *A. alpinum* are characterized by a relatively high variation and low gene flow between the analysed populations.
- Differences between patterns of morphological and isozyme variation are found in the studied species.

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Appendix 1. Collection sites of *Anthoxanthum odoratum*, *A. alpinum* and *A. aristatum* samples

Site no.	Locality, part of Poland	Geographical location (GPS)	Habitat type	Habitat abbr.	Collection date
		Anthoxanthum odoratum			
1	Jasionna I, Noteć Forest, W Poland	N52º46`42.6`` E16º27`05.5``	Pine forest	PF	21.05.2008
5	Torzym I, Rzepin Forest, W Poland	52º15'52.7"N 15º03'36.7"E	Roadside in pine forest	FR	29.05.2008
6	Torzym II, Rzepin Forest, W Poland	52º15'51.2"N 15º03'43.1"E	Roadside in pine forest	FR	29.05.2008
7	Debrznica I, Rzepin Forest, W Poland	52º14'27.6"N 15º02'28.4"E	Moist meadow	MM	29.05.2008
14	NW of Ruchocki Młyn, Nowy Tomyśl Sandur,W Poland	52º10'58.9"N 16º05'05.1"E	Pine forest	PF	14.05.2008
15	Ruchocki Młyn I, Nowy Tomyśl Sandur, W Poland	52º09'22.8"N 16º06'28.1"E	Moist meadow	MM	14.05.2008
16	Ruchocki Młyn II, Nowy Tomyśl Sandur, W Poland	52º09'22.0"N 16º06'25.5"E	Edge of pine forest	EF	14.05.2008
17	Chorzemin, Nowy Tomyśl Sandur, W Poland	52º09'10.7"N 16º06'49.9"E	Roadside in pine forest	FR	14.05.2008
18	Campus Morasko II, Poznań, W Poland	52º28'03.8"N 16º55'36.4"E	Sandy grassland near pine forest plantation	pG	07.05.2008
19	Campus Morasko III, Poznań, W Poland	52º28'08.0"N 16º55'33.8"E	Pine forest plantation	PP	12.05.2008
29	Babia Góra IV, S Poland	49º36'33.5"N 19º29'51.2"E	Lower montane meadow	LM	10.06.2011
37	Babia Góra II, S Poland	49º35'49.6"N 19º29'12.7"E	Lower montane meadow	LM	02.06.2008
		Anthoxanthum aristatum			
38	Nowa Tuchorza I, Nowy Tomyśl Sandur, W Poland	52º12'24.2"N 16º04'38.3"E	Arable field	A	14.05.2008
39	Wrzeszczyna II, Noteć Forest, W Poland	52º52'16.9"N 16º14'48.2"E	Sandy grassland near pine forest plantation	pG	21.05.2008
40	Gęstowice I, Rzepin Forest, W Poland	52º09'32.3"N 14º53'20.7"E	Fallow	F	29.05.2008
42	Wrzeszczyna I, Noteć Forest, W Poland	52º52'08.6"N 16º14'41.3"E	Arable field	A	21.05.2008
45	Chlebowo V, Noteć Forest, W Poland	52º44'51.4"N 16º45'59.8"E	Arable field	A	17.05.2007
46	Chlebowo VI, Noteć Forest, W Poland	52º44'51.4"N 16º45'59.8"E	Sandy grassland near pine forest	fG	17.05.2007
47	Chlebowo VII, Noteć Forest, W Poland	52º44'51.4"N 16º45'59.8"E	Field roadside	AR	17.05.2007
48	Chlebowo VIII, Noteć Forest, W Poland	52º44'51.4"N 16º45'59.8"E	Sandy grassland near arable field	aG	17.05.2007
53	Barłożnia, Nowy Tomyśl Sandur, W Poland	52º09'27.0"N 16º07'30.7"E	Sandy grassland near arable field	aG	14.05.2008
		Anthoxanthum alpinum			
55	Diablak (1697 m), Babia Góra, S Poland	49º34'19.3"N 19º31'48.4"E	Alpine grassland	AG	03.06.2008
56	Przełęcz Brona (1413 m), Babia Góra, S Poland	49º34'53.1"N 19º30'36.3"E	Subalpine matgrass meadow	SM	03.06.2008
57	Babia Góra III (1432 m), S Poland	49º34'49.0"N 19º30'43.4"E	Subalpine grassland near trail	SG	03.06.2008
58	Markowe Szczawiny (1166 m), Babia Góra, S Poland	49º35'21.6"N 19º31'06.9"E	Upper montane forest glade	UG	03.06.2011

Appendix 2. Mean values of genetic variability parameters for *Anthoxanthum alpinum* and *A. aristatum*, and band frequency for *A. odoratum* populations from investigated habitats

		Natural habitats						Seminatural habitats							
Character no.	Species	AG	SG	SM	UG	PF	OF	LM	MM	DM	fG	pG	aG	EF	PP
H_O	A.a.	0.294	0.298	0.401	0.513	-	-	-	-	-	-	-	-	-	-
	A.ar.	-	-	-	-	-	-	-	-	-	0.517	0.473	0.4195	-	-
H_E	A.a.	0.288	0.248	0.318	0.348	-	-	-	-	-	-	-	-	-	-
	A.ar.	-	-	-	-	-	-	-	-	-	0.423	0.436	0.401	-	-
F	A.a.	-0.025	-0.169	-0.215	-0.405	-	-	-	-	-	-	-	-	-	-
	A.ar.	-	-	-	-	-	-	-	-	-	-0.203	-0.08	-0.024	-	-
P%	A.a.	84.62	61.54	76.92	84.62	-	-	-	-	-	-	-	-	-	-
	A.ar.	-	-	-	-	-	-	-	-	-	100	100	100	-	-
	A.o.														
Pgi-1	1	-	-	-	-	0.88	-	0.82	0.82	-	-	0.73	-	0.87	0.83
	2	-	-	-	-	0.90	-	0.44	0.83	-	-	0.40	-	0.40	0.37
	3	-	-	-	-	0.40	-	0.56	0.33	-	-	0.80	-	0.53	0.70
Pgi-2	1	-	-	-	-	0.43	-	0.77	0.55	-	-	0.20	-	0.53	0.16
	2	-	-	-	-	0.29	-	0.23	0.28	-	-	0.40	-	0.00	0.10
	3	-	-	-	-	0.92	-	0.72	0.75	-	-	0.93	-	0.83	0.93
	4	-	-	-	-	0.71	-	0.68	0.85	-	-	0.067	-	0.90	0.60
	5	-	-	-	-	0.43	-	0.26	0.30	-	-	0.20	-	0.53	0.17
Idh-1	1	-	-	-	-	0.67	-	0.98	0.75	-	-	0.87	-	0.97	0.50
	2	-	-	-	-	0.38	-	0.93	0.48	-	-	0.13	-	0.03	0.50
	3	-	-	-	-	0.56	-	0.34	0.48	-	-	0.87	-	0.90	0.60
	4	-	-	-	-	1.00	-	0.70	0.92	-	-	1.00	-	1.00	1.00
	5	-	-	-	-	0.10	-	0.56	0.20	-	-	0.20	-	0.13	0.07
Px-1	1	-	-	-	-	0.44	-	0.44	0.65	-	-	0.60	-	0.43	0.60
	2	-	-	-	-	0.90	-	0.44	0.75	-	-	0.40	-	0.43	0.77
	3	-	-	-	-	0.75	-	0.47	0.47	-	-	0.60	-	0.73	0.70
	4	-	-	-	-	0.36	-	0.51	0.48	-	-	0.27	-	0.27	0.50
	5	-	-	-	-	1.00	-	0.70	0.73	-	-	1.00	-	0.73	0.73
*Px*2	1	-	-	-	-	0.43	-	0.79	0.35	-	-	0.13	-	0.47	0.97
	2	-	-	-	-	0.79	-	0.98	0.82	-	-	0.87	-	0.70	0.73
	3	-	-	-	-	0.56	-	0.98	0.83	-	-	0.80	-	0.93	0.97
Dia-1	1	-	-	-	-	0.98	-	0.75	0.60	-	-	0.87	-	0.90	0.80
	2	-	-	-	-	0.49	-	0.86	0.47	-	-	0.13	-	0.43	0.47
Dia-2	1	-	-	-	-	0.64	-	0.86	0.67	-	-	0.93	-	0.70	0.57
	2	-	-	-	-	0.78	-	0.49	0.97	-	-	1.00	-	0.93	0.60
	3	-	-	-	-	0.75	-	0.84	0.42	-	-	0.80	-	0.77	0.87
Got-1	1	-	-	-	-	0.82	-	0.68	0.68	-	-	0.40	-	0.90	0.60
	2	-	-	-	-	0.51	-	0.44	0.73	-	-	0.87	-	0.77	0.57
	3	-	-	-	-	0.49	-	1.00	0.15	-	-	0.60	-	0.43	0.57
Got-2	1	-	-	-	-	0.98	-	0.86	0.73	-	-	1.00	-	0.70	0.73
	2	-	-	-	-	0.58	-	0.42	0.25	-	-	0.33	-	0.90	0.97
	3	-	-	-	-	0.98	-	0.49	0.77	-	-	1.00	-	0.83	0.63
Pgd	1	-	-	-	-	0.58	-	0.77	0.53	-	-	0.40	-	0.73	0.97
	2	-	-	-	-	0.58	-	0.40	0.38	-	-	0.40	-	0.73	0.97
	3	-	-	-	-	0.70	-	0.58	0.20	-	-	0.77	-	0.87	0.73
Mdh	1	-	-	-	-	0.50	-	0.36	0.93	-	-	0.57	-	0.44	0.11
	2	-	-	-	-	0.47	-	0.48	0.28	-	-	0.34	-	0.70	0.47
	3	-	-	-	-	0.53	-	0.61	0.68	-	-	0.47	-	0.81	0.68
	4	-	-	-	-	0.38	-	0.66	0.72	-	-	0.43	-	0.77	0.45
	5	-	-	-	-	0.42	-	0.50	0.68	-	-	0.73	-	0.70	0.36
Pgm	1	-	-	-	-	0.78	-	0.49	0.62	-	-	0.23	-	0.35	0.68
	2	-	-	-	-	0.90	-	0.56	0.88	-	-	0.27	-	0.30	0.27
	3	-	-	-	-	0.27	-	0.62	0.48	-	-	0.67	-	0.14	0.17
Sdh	1	-	-	-	-	0.55	-	0.51	0.97	-	-	0.3	-	0.40	0.36
	2	-	-	-	-	0.50	-	0.45	0.93	-	-	0.57	-	0.78	0.55

Synanthropic habitats						
SR	LR	FR	AR	W	F	A
-	-	-	-	-	-	-
-	-	-	0.446	-	0.475	0.432
-	-	-	-	-	-	-
-	-	-	0.418	-	0.426	0.399
-	-	-	-	-	-	-
-	-	-	-0.034	-	-0.109	-0.043
-	-	-	-	-	-	-
-	-	-	90	-	91.67	93.257
-	-	0.90	-	-	-	-
-	-	0.49	-	-	-	-
-	-	0.49	-	-	-	-
-	-	0.37	-	-	-	-
-	-	0.31	-	-	-	-
-	-	0.87	-	-	-	-
-	-	0.51	-	-	-	-
-	-	0.11	-	-	-	-
-	-	0.80	-	-	-	-
-	-	0.34	-	-	-	-
-	-	0.60	-	-	-	-
-	-	0.96	-	-	-	-
-	-	0.24	-	-	-	-
-	-	0.66	-	-	-	-
-	-	0.54	-	-	-	-
-	-	0.52	-	-	-	-
-	-	0.32	-	-	-	-
-	-	0.90	-	-	-	-
-	-	0.41	-	-	-	-
-	-	0.37	-	-	-	-
-	-	0.81	-	-	-	-
-	-	0.73	-	-	-	-
-	-	0.52	-	-	-	-
-	-	0.79	-	-	-	-
-	-	0.94	-	-	-	-
-	-	0.40	-	-	-	-
-	-	0.68	-	-	-	-
-	-	0.53	-	-	-	-
-	-	0.43	-	-	-	-
-	-	0.67	-	-	-	-
-	-	0.64	-	-	-	-
-	-	0.70	-	-	-	-
-	-	0.81	-	-	-	-
-	-	0.81	-	-	-	-
-	-	0.61	-	-	-	-
-	-	0.59	-	-	-	-
-	-	0.47	-	-	-	-
-	-	0.40	-	-	-	-
-	-	0.73	-	-	-	-
-	-	0.70	-	-	-	-
-	-	0.76	-	-	-	-
-	-	0.82	-	-	-	-
-	-	0.62	-	-	-	-
-	-	0.68	-	-	-	-
-	-	0.59	-	-	-	-

Explanations: AG – alpine grassland, SG – subalpine grassland near trail, SM – subalpine matgrass meadow, UG – upper montane forest glade, PF – pine forest, OF – reed-grass oak forest, LM – lower montane meadow, MM – moist meadow, DM – dry meadow, fG – sandy grassland near pine forest, pG – sandy grassland near pine forest plantation, Ag – sandy grassland near arable field, EF – edge of pine forest, PP – pine forest plantation, SR – submontane ruderal roadside, LR – lower montane forest roadside, FR – roadside in pine forest, AR – field roadside, W – wasteland, F – fallow, A – arable field; A.a. – *Anthoxanthum alpinum*, A.ar. – *A. aristatum*, A.o. – *A. odoratum*; H_o – observed heterozygosity, H_e – expected heterozygosity, *F* – fixation index, P% – proportion of polymorphic loci

Appendix 3. Genetic diversity indices of *Anthoxanthum aristatum* populations

Population	Locus	*N*	*A*	N_e	H_o	H_e	*F*
39pG	*Pgi*-1	26	2	1.550	0.385	0.355	-0.083
	Pgi-2	26	4	3.388	0.846	0.705	-0.200
	Idh	26	2	1.742	0.385	0.426	0.097
	Px-1	26	2	1.929	0.423	0.482	0.121
	Px-2	26	2	1.649	0.385	0.393	0.023
	Dia-1	26	2	1.929	0.346	0.482	0.281
	Dia-2	26	2	1.304	0.269	0.233	-0.156
	Got-1	26	2	1.929	0.423	0.482	0.121
	Got-2	26	2	1.899	0.615	0.473	-0.300
	Pgd	26	2	1.550	0.308	0.355	0.133
	Mdh	26	2	1.899	0.692	0.473	-0.463
	Pgm	26	2	2.000	0.692	0.500	-0.385
	Sdh	26	2	1.451	0.385	0.311	-0.238
38A	*Pgi*-1	30	2	1.471	0.400	0.320	-0.250
	Pgi-2	30	3	2.410	0.533	0.585	0.088
	Idh	30	2	1.471	0.400	0.320	-0.250
	Px-1	30	2	2.000	0.400	0.500	0.200
	Px-2	30	2	1.600	0.433	0.375	-0.156
	Dia-1	30	2	1.301	0.200	0.231	0.135
	Dia-2	30	2	1.260	0.167	0.206	0.191
	Got-1	30	2	1.642	0.467	0.391	-0.193
	Got-2	30	2	1.867	0.333	0.464	0.282
	Pgd	30	1	1.000	-	-	-
	Mdh	30	3	1.909	0.633	0.476	-0.330
	Pgm	30	2	1.946	0.767	0.486	-0.577
	Sdh	30	2	1.923	0.733	0.480	-0.528
45A	*Pgi*-1	30	2	1.427	0.367	0.299	-0.224
	Pgi-2	30	3	2.597	0.633	0.615	-0.030
	Idh	30	2	1.301	0.267	0.231	-0.154
	Px-1	30	2	1.946	0.367	0.486	0.246
	Px-2	30	2	1.684	0.167	0.406	0.590
	Dia-1	30	2	1.642	0.400	0.391	-0.023
	Dia-2	30	2	1.557	0.333	0.358	0.068
	Got-1	30	2	1.867	0.400	0.464	0.139
	Got-2	30	2	1.946	0.567	0.486	-0.166
	Pgd	30	2	1.867	0.533	0.464	-0.148
	Mdh	30	2	1.800	0.600	0.444	-0.350
	Pgm	30	2	1.923	0.667	0.480	-0.389
	Sdh	30	2	1.642	0.467	0.391	-0.193
42A	*Pgi*-1	30	2	1.514	0.433	0.339	-0.277
	Pgi-2	30	4	3.315	0.700	0.698	-0.002
	Idh	30	2	1.923	0.667	0.480	-0.389
	Px-1	30	2	1.998	0.433	0.499	0.132
	Px-2	30	2	1.923	0.200	0.480	0.583
	Dia-1	30	2	1.385	0.267	0.278	0.040
	Dia-2	30	2	1.835	0.433	0.455	0.048
	Got-1	30	2	1.557	0.467	0.358	-0.304
	Got-2	30	2	1.301	0.200	0.231	0.135
	Pgd	30	3	1.145	0.100	0.127	0.211
	Mdh	30	3	2.113	0.333	0.527	0.367
	Pgm	30	3	2.113	0.533	0.527	-0.013
	Sdh	30	2	1.514	0.367	0.339	-0.080
40F	*Pgi*-1	10	2	1.600	0.500	0.375	-0.333
	Pgi-2	10	3	2.410	0.700	0.585	-0.197
	Idh	10	2	1.600	0.100	0.375	0.733
	Px-1	10	2	1.835	0.300	0.455	0.341
	Px-2	10	2	1.724	0.600	0.420	-0.429
	Dia-1	10	2	1.342	0.300	0.255	-0.176

Population	Locus	*N*	*A*	N_e	H_o	H_e	*F*
	Dia-2	10	2	1.835	0.100	0.455	0.780
	Got-1	10	1	1.000	-	-	-
	Got-2	10	2	1.980	0.500	0.495	-0.010
	Pgd	10	2	1.471	0.400	0.320	-0.250
	Mdh	10	2	1.923	0.800	0.480	-0.667
	Pgm	10	2	1.923	0.800	0.480	-0.667
	Sdh	10	2	1.724	0.600	0.420	-0.429
46fG	*Pgi*-1	18	2	1.857	0.722	0.461	-0.565
	Pgi-2	18	3	2.541	0.611	0.606	-0.008
	Idh	18	2	1.385	0.333	0.278	-0.200
	Px-1	18	2	1.385	0.222	0.278	0.200
	Px-2	18	2	1.528	0.333	0.346	0.036
	Dia-1	18	2	1.857	0.611	0.461	-0.324
	Dia-2	18	2	1.800	0.444	0.444	0.000
	Got-1	18	2	1.906	0.556	0.475	-0.169
	Got-2	18	2	1.528	0.444	0.346	-0.286
	Pgd	18	2	1.528	0.333	0.346	0.036
	Mdh	18	2	1.976	0.667	0.494	-0.350
	Pgm	18	2	1.857	0.722	0.461	-0.565
	Sdh	18	2	1.994	0.722	0.498	-0.449
47AR	*Pgi*-1	30	2	1.342	0.300	0.255	-0.176
	Pgi-2	30	3	2.663	0.767	0.624	-0.228
	Idh	30	2	1.514	0.433	0.339	-0.277
	Px-1	30	2	1.923	0.267	0.480	0.444
	Px-2	30	2	1.897	0.567	0.473	-0.199
	Dia-1	30	2	1.514	0.433	0.339	-0.277
	Dia-2	30	2	1.946	0.500	0.486	-0.029
	Got-1	30	2	1.471	0.333	0.320	-0.042
	Got-2	30	2	1.867	0.267	0.464	0.426
	Pgd	30	2	1.260	0.033	0.206	0.838
	Mdh	30	2	1.897	0.767	0.473	-0.622
	Pgm	30	2	1.980	0.700	0.495	-0.414
	Sdh	30	2	1.946	0.433	0.486	0.109
53aG	*Pgi*-1	30	2	1.600	0.500	0.375	-0.333
	Pgi-2	30	3	2.582	0.600	0.613	0.021
	Idh	30	2	1.897	0.767	0.473	-0.622
	Px-1	30	2	1.923	0.333	0.480	0.306
	Px-2	30	2	1.867	0.400	0.464	0.139
	Dia-1	30	2	1.301	0.200	0.231	0.135
	Dia-2	30	2	1.724	0.533	0.420	-0.270
	Got-1	30	2	1.385	0.200	0.278	0.280
	Got-2	30	2	1.142	0.133	0.124	-0.071
	Pgd	30	2	1.427	0.233	0.299	0.221
	Mdh	30	2	1.897	0.633	0.473	-0.340
	Pgm	30	2	1.897	0.700	0.473	-0.481
	Sdh	30	2	1.867	0.667	0.464	-0.435
48aG	*Pgi*-1	32	2	1.479	0.406	0.324	-0.255
	Pgi-2	32	4	2.809	0.656	0.644	-0.019
	Idh	32	2	1.438	0.250	0.305	0.179
	Px-1	32	2	1.909	0.406	0.476	0.147
	Px-2	32	2	1.983	0.406	0.496	0.180
	Dia-1	32	2	1.822	0.375	0.451	0.169
	Dia-2	32	2	1.909	0.406	0.476	0.147
	Got-1	32	2	1.319	0.281	0.242	-0.164
	Got-2	32	2	1.438	0.188	0.305	0.385
	Pgd	32	2	1.280	0.125	0.219	0.429
	Mdh	32	2	1.717	0.406	0.417	0.027
	Pgm	32	2	1.853	0.656	0.460	-0.425
	Sdh	32	2	1.822	0.438	0.451	0.030

Explanations: *N* – sample size, *A* – number of alleles per locus, N_e – effective number of alleles, H_o – observed heterozygosity, H_e – expected heterozygosity, *F* – fixation index