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 (*Nm* = 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

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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.
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 locus | Band |
|--------|--------|-------------|----------------------|-------------------|--------|----------------|--------|----------------|------|
| A      | PGI    | 5.3.1.9     | dimer                | Pgi-1             | 1,2    | Pgi-1          | 1,2    | Pgi-1          | 1,2  |
|        |        |             |                      | Pgi-2             | 1,2,3,4| Pgi-2          | 1,2,3,4| 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           | 1,2    | Px-2           | 1,2,3,4,5|
| GOT    |        | 2.6.1.1     | dimer                | Got-1             | 1,2    | Got-1          | 1,2    | Got-1          | 1,2,3,4,5|
|        |        |             |                      | Got-2             | 1,2    | Got-2          | 1,2,3  | Got-2          | 1,2,3,4,5|
| 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    | Mdh            | 1,2,3,4| Mdh            | 1,2,3,4,5|
|        | PGD    | 1.1.1.44    | dimer                | Pgd               | 1,2    | 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), phosphoglucuronase (PGM, EC 5.4.2.2), shikimate dehydrogenase (SHD, EC 1.1.1.25), 6-phosphoglucuronate dehydrogenase (PGD, EC 1.11.1.44), and malate dehydrogenase NAD⁺ (MDH, EC 1.11.37).

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 & Wendeen 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 (Świąńska 2008; Kuběsová 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 (χ²) test. The F-statistics helped to determine how genetic variation was distributed within and between populations. Gene flow (Nm) between populations was calculated from the formula: Nm = 0.25×(1-Fst)/Fst, where Fst 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 Fst 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). Hierarchical 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 Pogepene (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 \textit{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:

\[
S_{AB} = \frac{N_{AB}}{(N_A + N_B - N_{AB})},
\]

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 \textit{A. odoratum}, \textit{A. alpinum}, and \textit{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 \textit{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 \textit{A. odoratum} from diploid individuals of \textit{A. aristatum} and \textit{A. alpinum}.

\textbf{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, \textit{A. odoratum} can be distinguished from its diploid relatives: Pgi-2 in \textit{A. odoratum} has 1, 3 or 5 bands, whereas \textit{A. aristatum} has an additional, 4th allele at locus Pgi-2, and \textit{A. alpinum} has only 2 alleles at locus Pgi-2.

\textbf{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, \textit{A. odoratum} can be distinguished, as it has additional 4th and 5th bands, whereas diploid species have only 2 alleles at this locus.

\textbf{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 \textit{(Oryza sativa), Shahi et al. 1969} and next in common reed \textit{(Phragmites australis), Krazkowa 1996}.

\textbf{NAD 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 \textit{A. odoratum} can be distinguished from its diploid relatives.

\textbf{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.

\textbf{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
the diploid *A. aristatum*, an additional, 3<sup>rd</sup> allele was discovered at this locus, which makes it possible to distinguish the two species.

**Malate dehydrogenase NAD<sup>-</sup> (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 pine 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. 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).
Isocitrate dehydrogenase (IDH)

In this enzyme system, one locus \( \text{Idh-1} \) 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: \( \text{Px-1} \) and \( \text{Px-2} \) 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.

NAD diaphorase (DIA)

Activity of monomeric DIA was detected in 2 regions of the anodal part of the gel. Loci \( \text{Dia-1} \) and \( \text{Dia-2} \) had 2 alleles each. Their phenotypes were characteristic of dimeric enzymes: a single band for homozygotes and 3 bands for heterozygotes.

Glutamate oxaloacetic transaminase (GOT)

Activity of this enzyme was detected in 2 regions of the anodal part of the gel and labelled as \( \text{Got-1} \) and \( \text{Got-2} \). Both isozymes had 2 alleles each. Pheno types were characteristic of dimeric enzymes: a single band for homozygotes and 3 bands for heterozygotes.

Phosphogluconate dehydrogenase (PGD)

In the PGD system, one locus \( \text{Pgd} \) was found in the anodal part of the gel, with 3 alleles. Phenotypes were characteristic of monomeric enzymes.

Shikimate dehydrogenase (SHD)

One locus \( \text{Shd} \) 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 \( (\chi^2 \text{ test}) \). Allele frequency varied widely between the enzyme systems. \( \text{Pgd} \) 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: \( \text{Pgd} \) allele 2 and 3 and \( \text{Pgm-3} \) in population 42A and \( \text{Mdh} \) 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: \( \text{Pgd} \) and \( \text{Pgm} \) (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
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-
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
Table 7. Mean genetic diversity indices of *Anthoxanthum aristatum* populations

| Population | N  | A    | Ne   | P (%) | Ho   | He   | 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*|
| 46G        | 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, Ne – effective number of alleles, P – proportion of polymorphic loci, Ho – observed heterozygosity, He – expected heterozygosity, F – fixation index, * p ≤ 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

Fig. 8. UPGMA dendrogram for 9 *Anthoxanthum aristatum* populations, 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

### 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          |
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 $F_{ST}$, $F_{IS}$, 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 ($Pxd-1$ 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).

| 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 |
| $Pxd-1$ | 0.238 | 0.297 | 0.077 | 3.001 |
| $Pxd-2$ | 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 |

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

$F_{IS}$ = 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), * $p < 0.05$

**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: $Pxd-1$ in the anodal part of the gel, with 3 alleles, whereas $Pxd-2$ 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_e and H opportunistically 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_e and H_opportunistically 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 locus Px-1 and Pgd. In upper montane population 58UG (Markowe Szczawiny), small differences were found between H_e and H_opportunistically 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_Sit = 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 | 1      | 1.000| 1.000| 1.000| 1.000|
|       | 2      | 0.824| 0.786| 0.875| 0.417|
| Idh   | 1      | 0.176| 0.214| 0.125| 0.583|
|       | 2      | 0.647| 0.714| 0.250| 0.583|
|       | 3      | 0.324| 0.250| 0.750| 0.000|
| Px-1  | 1      | 1.000| 1.000| 1.000| 1.000|
|       | 2      | 0.088| 0.071| 0.000| 0.167|
| Dia-1 | 1      | 0.912| 0.929| 1.000| 0.833|
|       | 2      | 0.059| 0.000| 0.000| 0.250|
| Got-1 | 1      | 0.941| 1.000| 1.000| 0.750|
|       | 2      | 0.088| 0.107| 0.000| 0.583|
|       | 3      | 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|
| 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 ($H_o$), expected heterozygosity ($H_e$) 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<sub>e</sub> | H<sub>o</sub> | H<sub>e</sub> | 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<sub>e</sub> – number of effective alleles, H<sub>o</sub> – observed heterozygosity, H<sub>e</sub> – expected heterozygosity, F – fixation index, 55AG – Diab-łak, 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<sub>e</sub> | P (%) | H<sub>o</sub> | H<sub>e</sub> | 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<sub>e</sub> – number of effective alleles, P – proportion of polymorphic loci, H<sub>o</sub> – observed heterozygosity, H<sub>e</sub> – 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 ≤ 0.05*

Fig. 10. Mean values observed heterozygosity (H<sub>o</sub>), expected heterozygosity (H<sub>e</sub>) 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<sub>is</sub> | F<sub>st</sub> | F<sub>it</sub> | N<sub>m</sub> |
|-------|----------------|-------------|-------------|-------------|
| 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<sub>is</sub> – inbreeding coefficient, F<sub>st</sub> – total inbreeding coefficient, F<sub>it</sub> – coefficient of genetic differentiation between populations, N<sub>m</sub> – rate of gene flow between populations, N<sub>m</sub> = [(1/F<sub>st</sub>) - 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
3.4. Morphological versus isozyme variation of *Anthoxanthum odoratum, A. alpinum*, and *A. aristatum*

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.

3.4.1. Morphological versus isozyme variation of *Anthoxanthum odoratum*

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. 11. UPGMA dendrogram of *Anthoxanthum alpinum* populations, based on Nei’s (1978) genetic distances](image)

Explanations: site numbers – see Appendix 1

![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](image)

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

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**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

**Fig. 14.** Spearman rank correlation between Nei’s (1978) genetic distances and Mahalanobis distances for *Anthoxanthum aristatum* 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* = 0.301 in the present study is higher than *H* = 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*<sub>ST</sub> = 0.061 for *Got*-1, *F*<sub>ST</sub> = 0.371 for *Got*-2, *F*<sub>ST</sub> = 0.229 for *Px*-1, *F*<sub>ST</sub> = 0.310 for *Px*-2, and *F*<sub>ST</sub> = 0.094 for *Pgd*-1 (Table 14). Mean genetic differentiation among populations was relatively high (*F*<sub>ST</sub> = 0.198), and gene flow was relatively low (*N*<sub>m</sub> = 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 & Piekoś-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.
- *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 |
|----------|--------------------------|-----------------------------|--------------|---------------|----------------|
| 1        | Jasionna I, Noteć Forest, W Poland | 52°14′46″.2″N 15°02′05.5″E | Pine forest | PF | 21.05.2008 |
| 5        | Torzym I, Rzepin Forest, W Poland | 52°15′52.7″N 15°03′36.7″E | Roadside in pine forest | FR | 29.05.2008 |
| 6        | Torzym II, Rzepin Forest, W Poland | 52°15′51.2″N 15°03′43.1″E | Roadside in pine forest | FR | 29.05.2008 |
| 7        | Debrznica I, Rzepin Forest, W Poland | 52°14′27.6″N 15°02′28.8″E | Moist meadow | MM | 29.05.2008 |
| 14       | NW of Ruchocki Młyn, Nowy Tomyśl Sandur, W Poland | 52°10′58.9″N 16°05′05.1″E | Pine forest | PF | 14.05.2008 |
| 15       | Ruchocki Młyn I, Nowy Tomyśl Sandur, W Poland | 52°09′22.8″N 16°06′28.1″E | Moist meadow | MM | 14.05.2008 |
| 16       | Ruchocki Młyn II, Nowy Tomyśl Sandur, W Poland | 52°09′22.0″N 16°06′25.5″E | Edge of pine forest | EF | 14.05.2008 |
| 17       | Chorzemin, Nowy Tomyśl Sandur, W Poland | 52°09′10.7″N 16°06′49.9″E | Roadside in pine forest | FR | 14.05.2008 |
| 18       | Campus Morasko II, Poznań, W Poland | 52°28′03.8″N 16°55′36.4″E | Sandy grassland near pine forest plantation | pG | 07.05.2008 |
| 19       | Campus Morasko III, Poznań, W Poland | 52°28′08.0″N 16°55′33.8″E | Pine forest plantation | PP | 12.05.2008 |
| 29       | Babia Góra IV, S Poland | 49°36′33.5″N 19°29′51.2″E | Lower montane meadow | LM | 10.06.2011 |
| 37       | Babia Góra II, S Poland | 49°35′49.6″N 19°29′12.7″E | Lower montane meadow | LM | 02.06.2008 |
| 38       | Nowa Tuchorza I, Nowy Tomyśl Sandur, W Poland | 52°12′24.2″N 16°04′38.3″E | Arable field | A | 14.05.2008 |
| 39       | Wrzeszczyna II, Noteć Forest, W Poland | 52°52′16.9″N 16°14′48.2″E | Sandy grassland near pine forest plantation | pG | 21.05.2008 |
| 40       | Gęstowice I, Rzepin Forest, W Poland | 52°09′32.3″N 14°53′20.7″E | Fallow | F | 29.05.2008 |
| 41       | Wrzeszczyna I, Noteć Forest, W Poland | 52°52′08.6″N 16°14′41.3″E | Arable field | A | 21.05.2008 |
| 42       | Chlebowo V, Noteć Forest, W Poland | 52°44′51.4″N 16°45′59.8″E | Arable field | A | 17.05.2007 |
| 43       | Chlebowo VI, Noteć Forest, W Poland | 52°44′51.4″N 16°45′59.8″E | Sandy grassland near pine forest | fG | 17.05.2007 |
| 44       | Chlebowo VII, Noteć Forest, W Poland | 52°44′51.4″N 16°45′59.8″E | Field roadside | AR | 17.05.2007 |
| 45       | Chlebowo VIII, Noteć Forest, W Poland | 52°44′51.4″N 16°45′59.8″E | Sandy grassland near arable field | aG | 17.05.2007 |
| 53       | Barłożnia, Nowy Tomyśl Sandur, W Poland | 52°09′27.0″N 16°07′30.7″E | Sandy grassland near arable field | aG | 14.05.2008 |

### Anthoxanthum aristatum

| Site no. | Locality, part of Poland | Geographical location (GPS) | Habitat type | Habitat abbr. | Collection date |
|----------|--------------------------|-----------------------------|--------------|---------------|----------------|
| 55       | Diablak (1697 m), Babia Góra, S Poland | 49°34′19.3″N 19°31′48.4″E | Alpine grassland | AG | 03.06.2008 |
| 56       | Przegłęcz Brona (1413 m), Babia Góra, S Poland | 49°34′53.1″N 19°30′36.3″E | Subalpine matgrass meadow | SM | 03.06.2008 |
| 57       | Babia Góra III (1432 m), S Poland | 49°34′49.0″N 19°30′43.4″E | Subalpine grassland near trail | SG | 03.06.2008 |
| 58       | Markowe Szczawiny (1166 m), Babia Góra, S Poland | 49°35′21.6″N 19°31′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

| Character no. | Species | AG | SG | SM | UG | PF | OF | LM | MM | DM | fG | pG | aG | EF | PP |
|---------------|---------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| \(H_0\)      | A.a.    | 0.294 | 0.298 | 0.401 | 0.513 | - | - | - | - | - | - | - | - | - | - |
|               | A.arr.  | -   | -   | -   | -   | - | - | - | - | - | - | - | - | - | - |
| \(H_e\)      | A.a.    | 0.288 | 0.248 | 0.318 | 0.348 | - | - | - | - | - | - | - | - | - | - |
|               | A.arr.  | -   | -   | -   | -   | - | - | - | - | - | - | - | - | - | - |
| \(F\)        | A.a.    | -0.025 | -0.169 | -0.215 | -0.405 | - | - | - | - | - | - | - | - | - | - |
|               | A.arr.  | -   | -   | -   | -   | - | - | - | - | - | - | - | - | - | - |
| \(P%\)       | A.a.    | 84.62 | 61.54 | 76.92 | 84.62 | - | - | - | - | - | - | - | - | - | - |
|               | A.arr.  | -   | -   | -   | -   | - | - | - | - | - | - | - | - | - | - |
|               | A.o.    | -   | -   | -   | -   | - | - | - | - | - | - | - | - | - | - |

\[\begin{array}{cccccccccccccccc}
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 \\
P5-1  & 1 & - & - & - & - & 0.44 & - & 0.93 & 0.48 & - & - & 0.13 & - & 0.03 & 0.50 \\
   & 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 \\
P5-2  & 1 & - & - & - & - & 0.43 & - & 0.90 & 0.26 & - & - & 0.00 & - & 0.73 & 0.73 \\
   & 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 \\
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 \\
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 & - & 0.54 & 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 \\
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 
\end{array}\]
**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 | -  |

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 raderal 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  |
|------------|-------|----|----|-----|-----|-----|----|
| 39G        | 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.