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MORPHOLOGICAL AND ANATOMICAL VARIABILITY OF ISOZYMATICALLY IDENTIFIED CLONES OF *PINUS MUGO* TURRA

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This study examined the anatomical and morphological variability of 10 needle traits in isozymatically identified clones of three peatbog populations of *Pinus mugo*, focusing on variation within and between clones, and the relation between isozyme variation and morpho-anatomical characters of needles. In each peatbog there were clones exhibiting high and low plasticity of the anatomical and morphological traits studied. In general, three types of variation within clones were distinguished: (1) clones with ramets very similar to each other, (2) clones with extensive intra-clone variability, and (3) clones with intermediate variability. The differences in phenotypic variability within clones may be explained by differences in the reaction norm of ramets in particular clones and by somatic mutations. In respect to anatomical, morphological and isozymatic traits, clones from the same peatbog showed more similarity to each other than to clones from other peatbogs.

Key words: Dwarf mountain pine, needle anatomy and morphology, phenotypic plasticity, reaction norm.

INTRODUCTION

In studies of the diversity of clonal plants, two levels of organization have to be considered: genets and ramets (Harper, 1977). A genet represents all tissue originating from one zygote, and a ramet is an independent or potentially independent part of a genet. The number of ramets per genet determines the clonal diversity of a given population.

Dwarf mountain pine (*Pinus mugo* Turra) is widely distributed in the Tatra Mts in Poland. It is adapted to difficult environmental conditions, including poor oligotrophic peatbogs in the mountains. Aside from sexual reproduction it can reproduce asexually by rooting shoots (Seneta and Dolatowski, 1997). For this and many other species, vegetative reproduction has adaptive significance.

Because of phenotypic plasticity, morphological characters cannot be easily used to identify an individual genet and the corresponding clone. However, the first study on the variability of *P. mugo* clones, from a peatbog below Ostry Wierch Mt. in the Tatras, offered evidence to challenge this view (Bączkiewicz and Prus-Głowacki, 1997).

In the present study we analyzed anatomical and morphological variability in clones earlier identified isozymatically from three peatbogs: Wielka Pańszczycka Młaka (WPM), Waksmundzka Młaka (WM), and Dolina Kościeliska (DK) in the Tatra Mts. Morphological studies on clones, which very often consist of many distinct plants (ramets) of the same origin, enable plasticity within a clone to be observed. Anatomical and morphological variability between ramets within the same clone may also be used to estimate the degree of environmental modification of the phenotype. In the present investigation we attempted to answer the following questions:

(1) What is the range of anatomical and morphological intra-clone variability?
(2) Are the inter-clone differences in needle anatomy and morphology plain enough to identify the clones on the basis of the traits?
(3) Is isozyme variation related to the morpho-anatomical characters of needles in the studied clones?

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TABLE 1. Geographic coordinates, altitiude, and number of clones and ramets in the studied populations of *Pinus mugo* Turra

Peatbog	Geographic coordinates	Altitude (m)	No. of clones	Clone numbers (and no. of ramets)
Wielka Pańszczycka Młaka (WPM)	49°13'35"N 19°52'00"E	1260	2	1 (7), 2 (6)
Polana Waksmundzka (WM)	49°16'10"N 20°02'30"E	1380	4	3 (8), 4 (5), 5 (8), 6 (7)
Dolina Kościeliska (DK)	49°15'30"N 20°03'07"E	1100	16	7 (5), 8 (13), 9 (8), 10 (11), 11 (13), 12 (7), 13 (5), 14 (9), 15 (6), 16 (6), 17 (5),18 (5), 19 (6), 20 (5), 21 (8), 22 (6)

MATERIALS AND METHODS

Winter buds and two-year-old needles were collected from three *P. mugo* populations colonizing three peatbogs in the Tatra Mountains in Poland: Dolina Kościeliska (DK), Wielka Pańszczycka Młaka (WPM), and Waksmundzka Młaka (WM) (Tab. 1). From each population, samples were collected every 3 m along a transect. The number of collected samples (300 from DK, 86 from WPM, 64 from WM) depended on the population size.

The winter buds were used in isozymatic analysis for clone identification, using the methods for horizontal starch-gel electrophoresis, staining procedure and isozyme assay described by Rudin and Ekberg (1978), Szmidt and Yazdani (1984), Yazdani et al. (1985) and Gullberg et al. (1985). The following enzyme systems were analyzed: two loci of glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1); alcohol dehydrogenase (ADH, E.C. 1.1.1.1); diaphorase (DIA, E.C. 1.8.1.4); fluorescent esterase (FEST, E.C. 3.1.1.23); glutamate dehydrogenase (GDH, E.C. 1.4.1.2); glucose–6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.44); two loci of malate dehydrogenase (MDH, E.C.1.1.1.37); and two loci of shikimate dehydrogenase (ShDH, E.C.1.1.1.25). Samples that demonstrated the same genotype in all 11 examined loci were regarded as representing ramets of a single clone. The formula of Aspinwall and Christan (1992) was used to calculate the probability that ramets with the same multilocus pattern belonged to the same clone. For biometric analyses we selected clones represented by at least 5 ramets, to have a proper sample for checking intra-clone variability (Tab. 1). To standardize the conditions of biometric analysis, the 5 ramets were selected at random from a clone consisting of more than 5 ramets.

Two-year-old needles were used for anatomical and morphological investigations. From each sample (ramet), 30 needles were chosen randomly and preserved in 70% ethyl alcohol for about one year. First the morphological traits were studied, then each needle was cut transversely at the midpoint of its length. The obtained sections for anatomic analysis were embedded in polyvinyl alcohol. In total, 660 needles were examined. Ten quantitative traits were assessed (Tab. 2).

TABLE 2. Needle traits measured in ramets of *Pinus mugo* Turra clones from three peatbog populations (WPM, WM, DK) in the Tatra Mts

No.	Traits
1.	Number of stomatal rows on abaxial (convex) side of needle
2.	Number of stomatal rows on adaxial (flat) side of needle
3.	Mean number of stomata in 2-mm-long section of abaxial side of needle
4.	Mean number of stomata in 2-mm-long section of adaxial side of needle
5.	Number of resin canals
6.	Thickness of epidermis on adaxial side of needle (µm)
7.	Mean width of epidermal cells, measured as average width of 3 neighboring cells (µm)
8.	Width of needle in cross-section (µm)
9.	Thickness of needle in cross section (µm)
10.	Distance between vascular bundles (µm)

The data obtained (untransformed) were analyzed statistically with STATISTICA 5.5 for Windows (StatSoft Inc.). Descriptive statistics (arithmetic means, standard deviations, minima and maxima) and coefficients of variation were computed to evaluate the range of variation of anatomical and morphological traits (Williams, 1995; Łomnicki, 1999). The standard method of discriminant analysis (Caliński and Kaczmarek, 1973; Caliński et al., 1975; Krzyśko, 1990), Mahalanobis distances between ramets within clones and between clones, and the T^2 Hotelling test (Hotelling, 1957; Krzyśko, 1982, 1990;) were applied. To limit the overall experimentwise error rate α, the Bonferroni correction for critical T^2 Hotelling value at $\alpha = 0.05$, $\alpha' = \alpha / k$, was used, where k is the number of intended tests (Sokal and Rohlf, 1997). The complete linkage method of principal component analysis (Sneath and Sokal, 1973; Caliński et al., 1975) was used to compare the differences between clones and ramets within a single clone.

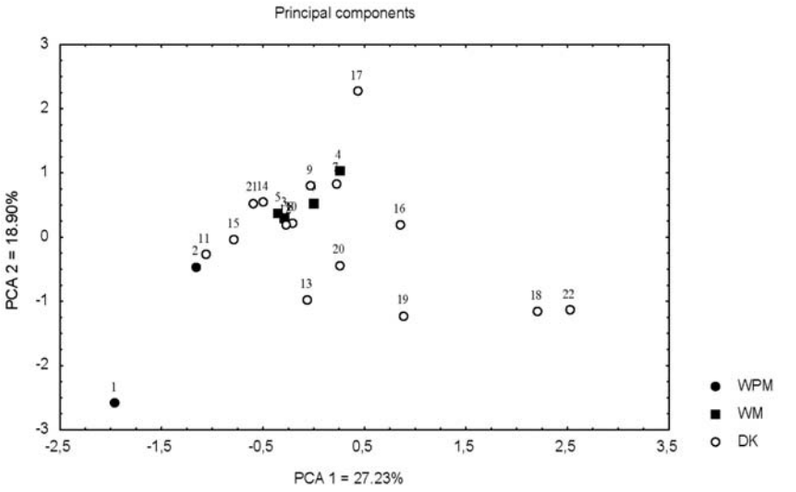


Fig. 1. Distribution of the 22 studied clones of *Pinus mugo* Turra on the plane of the first and second principal component axes, based on 11 isozyme loci.

RESULTS

ISOZYMATIC VARIABILITY

In all, 215 clones were found in the studied populations (63 at WPM, 25 at WM, 127 at DK). The identified clones differed in size; 22 clones had at least 5 ramets and were used for biometric analyses: 2 clones from WPM, 4 from WM and 16 from DK (Tab. 1). The average probability that ramets with the same isozymatic pattern belonged to the same clone was 95%. Figure 1 is a principal component scatter diagram based on the multilocus genotypes of 22 clones, showing that clone no. 1 from WPM is distinct from the other clones. The clones from WM (3, 4, 5, 6) were positioned close to each other, indicating their genetic similarity. The clones from DK formed a loose, internally differentiated cluster.

MORPHOLOGY

Characteristics of the studied traits

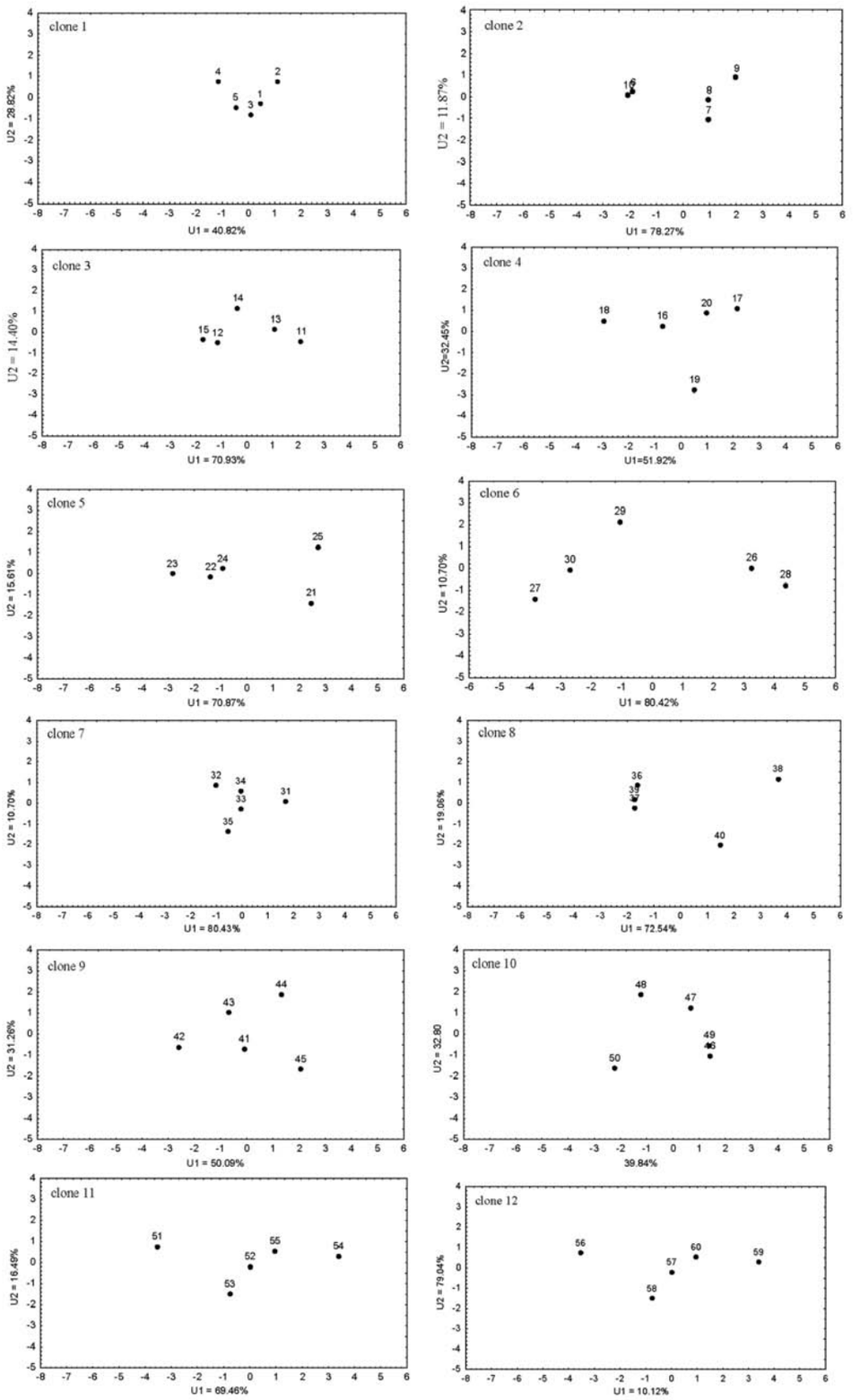
In the 22 clones examined, the lowest coefficient of variation was for distance between vascular bundles (trait 10) in all the studied clones (5.5% to 6.2%). The highest coefficient of variation was for number of resin canals in the DK samples (24 .9%) (Tab. 3). The clones from WPM varied least. For all the examined clones, the strongest inter-trait correlation was between needle width and thickness measured in cross section (traits 8 and 9). In 18 clones, trait 8 was strongly correlated with five other traits (1, 2, 5, 9, 10).

Intra-clone variability

Figure 2 gives the results of discriminant analysis for 5 ramets within each studied clone of *P. mugo* in the space of the first two discriminant variables. The mean Mahalanobis distances between ramets for each clone are shown in Figure 3. The critical value of the T^2 Hotelling test with Bonferroni correction was $T^2_{0.005}$ = 435.36. Analyses of the discriminant variables and the diagram of mean Mahalanobis distances indicate low to very high variability between ramets of individual clones. In general, three types of variation within individual clones might be distinguished: (1) clones with

TABLE 3. Coefficient of variation of 10 studied anatomical and morphological needle traits in the three examined groups of clones from peatbogs (WPM, WM, DK)

Peatbog	Traits									
	1	2	3	4	5	6	7	8	9	10
Wielka Pańszczycka Młaka (WPM)	14.8	14.0	7.8	6.7	18.3	14.5	12.1	7.9	5.7	5.5
Waksmundzka Młaka (WM)	19.2	23.8	8.3	9.5	22.6	14.0	11.2	9.1	10.3	5.7
Dolina Kościeliska (DK)	20.5	18.3	9.8	9.6	24.9	13.8	11.7	8.5	8.5	6.2


clone 1
U2 = 28.82%
U1 = 40.82%
clone 2
U2 = 11.87%
U1 = 78.27%
clone 3
U2 = 14.40%
U1 = 70.93%
clone 4
U2=32.45%
U1=51.92%
clone 5
U2 = 15.61%
U1 = 70.87%
clone 6
U2 = 10.70%
U1 = 80.42%
clone 7
U2 = 10.70%
U1 = 80.43%
clone 8
U2 = 19.06%
U1 = 72.54%
clone 9
U2 = 31.26%
U1 = 50.09%
clone 10
U2 = 32.80
39.84%
clone 11
U2 = 16.49%
U1 = 69.46%
clone 12
U2 = 79.04%
U1 = 10.12%

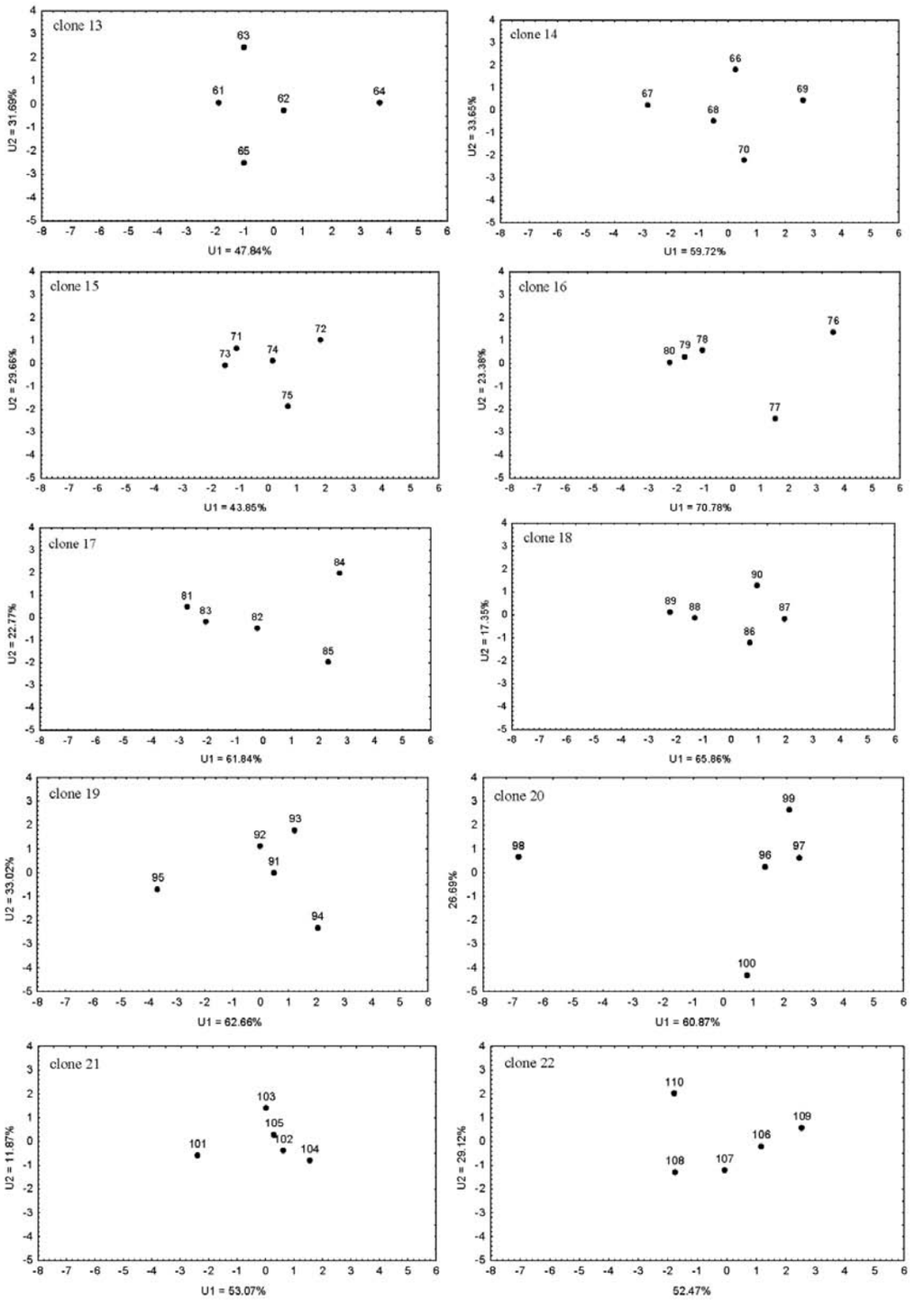


Fig. 2. Results of discriminant analysis of 5 ramets of each of 22 clones of *Pinus mugo* Turra for the first two discriminant variables (U_1, U_2) of the applied set of 10 anatomical and morphological needle traits.

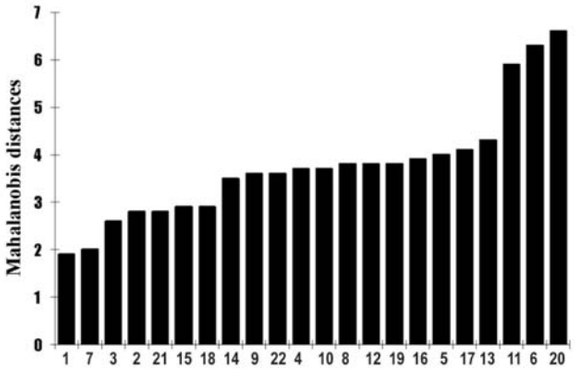


Fig. 3. Mean Mahalanobis distances between ramets of each of 22 studied clones of *Pinus mugo* Turra.

ramets very similar to each other (clones 1, 7), with no statistically significant Mahalanobis distances; (2) clones strongly differentiated (6, 11, 20), with most calculated Mahalanobis distances greater than the critical value ($T^2_{0.005}$); and (3) clones with intermediate variation.

Inter-clone variability

Inter-clone variability is illustrated by a scatter diagram (Fig. 4) on the plane of the first two principal components. Together the components accounted for 62% of the variability of the trait set. The pattern of the diagram is very similar to the result from principal component analysis based on enzymatic data (Fig. 1). Clone 1 from WPM was distinct from the rest, and the clones from WM occupied an intermediate position between the WPM and DK populations.

DISCUSSION

In this study, low as well as high plasticity was revealed among the selected clones studied. This is best exemplified by the DK peatbog, where clone 7 (with morphologically very similar ramets) grows next to clone 20 (with highly variable ramets). Similarly, at the WM peatbog, clone 3 with low inter-ramet plasticity was found close to the highly plastic clone 6 (Figs. 2, 3). In general, three types of variability were distinguished within individual clones of mountain dwarf pine: (1) clones with ramets very similar in terms of the traits studied, (2) clones with extensive intra-clone variability, and (3) clones with intermediate variability.

Phenotypic variability within a clone may result from the modifying effect of the environment (different degrees of exposure to sunlight, different soil conditions, etc.) (e.g., Pigliucci, 1996; Morabito et al., 1996; Hutchings and de Kroon, 1994; Navas and Garnier, 2002), and also from genetic factors such as somatic mutations (Klekowski, 1988; Guttman, 1997; Orive, 2001). Many studies of morphological clonal variability have found, even within single clones, a capacity for considerable change in morphology in the context of local variation of abiotic and biotic conditions (Mitton and Grant, 1980; Schmid, 1990; Douglas, 1991; Turkington et al., 1991; Hutchings and de Kroon, 1994; Sipes and Wolf, 1997). The ramets of the studied clones of dwarf mountain pine grew next to each other in natural populations on peatbogs. Environmental conditions are similar but not identical between sites, and the ramets differ in age, size, position within the cluster, etc. According to Schmid (1990), high phenotypic variability between ramets is common. In some cases this may result from competition between ramets for nutrients (Falconer, 1960). This is particularly evident in areas of significant spatial differentiation and at sites where large changes occur in the environment during the plant's life (Levins, 1968). Many authors have noted that ramets of the same clone can develop distinct morphological forms, depending on environmental conditions. For example, in a study of *Salix setchallina*, Douglas (1991) described changes in ramet forms during the lifespan of an individual.

Every individual has a specific norm of reactions to environmental factors, that is, it has a capacity for certain morphological modifications within a specific range. Plants of the same genotype (as in the case of clones) should exhibit the same reaction norm (Szmalhauzen, 1975; Pigliucci, 1996). Unlike the reaction norm, environmentally induced variability itself is not inherited. The differences in the extent of morphological variability within the studied clones may be explained by differences in the range of reaction norms between clones. Individuals with a wider reaction norm are more variable (e.g., clones 6, 11, 20) than those with a narrow reaction norm (e.g., clones 1, 7).

Somatic mutations in the apical cells of meristematic buds, from which new ramets can form, may be an important source of intra-clone variability. Most somatic mutations are presumed to cause no major change but are genetically detectable (Silander, 1985). A frequency of such mutations in the range of 10^{-3}–10^{-5} per locus has been reported for higher plants (Antion and Strobeck, 1985). In the case of old clones, the accumulated somatic mutations may shape the variability within clones (Silander, 1979). In stable environmental conditions, clone age may reach hundreds or even thousands of years. Some clones of American poplar are estimated to be around 10,000 years old (Kemperman and Barnes, 1976). The dwarf mountain pine in the Tatra Mts peatbogs may be a relict of the glacial age (Środoń, 1959). The age of peatbogs in the Tatra Mountains is estimated at 5,000 to 10,000 years (Obidowicz, 1975). Thus, some *P. mugo* clones growing

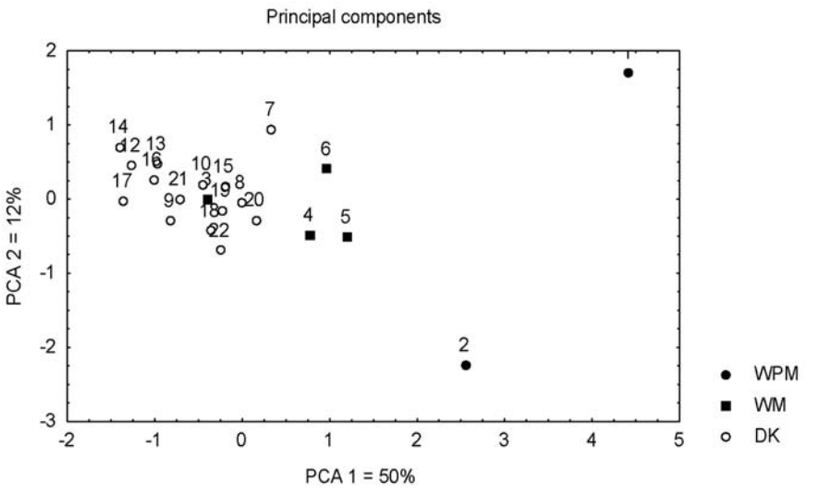


Fig. 4. Results of principal component analysis for 22 clones of *Pinus mugo* Turra from three peatbog populations on the plane of the first two principal components from the applied set of 10 morphological and anatomical needle traits.

on peatbogs may be several thousand years old, enough time for many somatic mutations to accumulate within a single clone. Thus in *P. mugo*, the accumulated somatic mutations, competition and the modifying effect of the environment are most probably the direct causes of the variability within clones, but the range of the phenotypic variability within a clone is conditioned by its genetic reaction norm.

Clones from the same peatbog resembled each other with respect to anatomical, morphological and enzymatic traits, and clones from different peatbogs exhibited more divergence (Figs. 1, 4). The similarity between clones within a single peatbog may be linked not only to the genetic relationships between individuals but also to the similarity of environmental conditions. The dwarf mountain pine populations on the peatbogs are small, consisting of small numbers of individuals surrounded by high spruce trees. They are distant from the continuous range of dwarf mountain pine. Thus the studied populations are subject to inbreeding because of the limited gene inflow.

The narrowest range of variability (anatomical, morphological and isozymatic) was noted at the WM peatbog, as demonstrated by the results of principal component analysis (Figs. 1, 4). The high similarity between clones from the WM peatbog probably was due to its small population size, compared to those at the other studied peatbogs. In terms of needle anatomy and morphology, the two clones from the WPM peatbog distinctly differed from the clones at WM and DK (Fig. 5). Both clones, but particularly clone 1, showed low intra-clone plasticity.

The patterns in the graphs illustrating enzymatic traits and anatomical and morphological measurements resemble each other (Figs. 1, 4). This attests to a correlation between enzymatic traits and morphological characters. In *Pinus sylvestris*, such a correlation has been reported by Oleksyn et al. (1994), Prus-Głowacki et al. (1998), and Prus-Głowacki et al. (1999) for several phenotypic traits including tree size, decrease in annual growth rate, mortality and genetic structure.

Biometric analyses showed that the *P. mugo* clones from the studied peatbogs cannot be clearly distinguished from each other on the basis of anatomical and morphological traits alone. Such identification was possible only when the clones exhibited very distinct characters, as in similar studies of clones from the Wyżnia Pańszczycka Młaka peatbog (Bączkiewicz and Prus-Głowacki, 1997). Clones can be distinguished more precisely if molecular traits are used. Analysis of biometric characters provides only additional information about the clones and about the modifying effect of the environment, and also helps establish the range of their reaction norms.

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