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**THE USE OF AFLP MARKERS IN CONSERVATION GENETICS
– A CASE STUDY ON *PULSATILLA VERNALIS* IN THE POLISH
LOWLANDS**

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Abstract: *Pulsatilla vernalis* is a rare species in the Polish lowlands, strongly threatened by anthropogenic disturbance of its habitats. A grave decrease in its populations has been observed during the past 60-80 years (analogous populations in Eastern Austria and the Czech Republic are almost or completely extinct). An analysis of the genetic diversity of populations in the Polish lowlands was performed to estimate its level and distribution. The AFLP method was used for the study of seven populations. An analysis using five pairs of selective primers revealed 446 scorable fragments; 62.1% of them were polymorphic. The average gene diversity indices was 0.17 (the mean value for all the populations), ranging from 0.139 to 0.204. A weak relationship between diversity and population size was revealed. Most of the genetic diversity was contributed to by the within-population level (AMOVA) and only a weak geographical structure was shown by UPGMA clustering. Four populations formed population-specific clusters while three others (from one region) were intermixed. These preliminary results show a moderate genetic diversity of the studied populations, which was still rather high when compared with their size. This result, together with the low between-population differentiation in the region, suggests that these populations are the remnants of larger populations that, only a few decades ago, were much less isolated.

Key Words: *Pulsatilla vernalis* (L.) Mill., Ranunculaceae, AFLP, Genetic Diversity, Conservation, Poland.

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INTRODUCTION

Pulsatilla vernalis (Ranunculaceae) is a species endemic to Europe, mainly found in the Western European mountains (Alps, Pyrenees), where it is common. However, it also has a lowland distribution in Central and Northern Europe [1]. The populations in the Central European lowlands are considered to be relictual alpine-boreal elements [2]. These lowland populations are very fragile and rarely exceed the size of a few dozen individuals [3]. The plant is extinct in its lowland habitats in Eastern Austria and almost extinct in the Czech Republic [4]. A constant decrease in the *P. vernalis* populations has also been observed in Poland for the past 60-80 years, and the plant is classified as 'vulnerable' (Vu) in the Polish Red Data Book of Plants [5]. An analysis of the genetic diversity of an endangered plant species is one of the key elements in the estimation of the viability of its populations, and in the establishment of efficient *in situ* as well as *ex situ* preservation [6]. In the case of *Pulsatilla vernalis*, the AFLP (Amplified Fragment Length Polymorphism [7]) technique was used for the assessment of genetic diversity. The usefulness of this marker type in conservation genetics despite its dominant character – it gives rapid generation of large numbers of fragments – is discussed in several papers [eg. 8, 9]. The preliminary results of the analysis of the genetic diversity level of *P. vernalis* in the Polish lowlands, and the spatial distribution of this diversity are reported on in this paper, together with some initial conclusions regarding the conservation of this rare species.

MATERIALS AND METHODS

Plant material

Samples of *P. vernalis* were collected in May-June, 2001, from seven populations covering different regions of plant distribution in the Polish lowlands. Single young leaves were cautiously removed, immediately put into bags with silica gel and stored at room temperature. Sampling depended upon the population size and included 2-15 specimens per population (see Tab. 1). In total, 58 samples were used for molecular analysis (a few samples that failed to amplify are excluded from the calculation).

AFLP analysis

DNA extraction, using ca. 30 mg of dried leaf material, was performed using the DNeasy Plant Mini Kit (QIAGEN), according to a slightly modified version of the manufacturer's protocol (additional washing steps were applied), and included a subsequent additional purification using the Wizard DNA Clean-up System (Promega). The principles of the AFLP procedure are given in [7]. *EcoRI* and *Tru9I* (= *MseI*) restriction enzymes were used for digestion. Preselective PCR was performed using *EcoRI* (A) and *MseI* (C) preselective primers. For selective PCR, 32 primer combinations were tested on six

individuals, and five were chosen for final analysis (*EcoRI* and *MseI* primers with three and four selective nucleotides respectively), based on the clarity of

Tab. 1. Location of the populations studied, estimated population size (S), number of individuals analysed (N), number and proportion of polymorphic loci in the population for five individuals per population (except for population 5) (P), number and proportion of polymorphic loci in the total population sampling (P_T), number of private fragments, confined to only one population (U), average gene diversity in the population calculated for five individuals per population (except for population 5) (D).

No	Location	S	N	P	P _T	U	D
1	Dołki (vicinity of Kielce) N 50° 48'20" E 20°18'30"	30	10	155 (35%)	191 (43%)	1	0.167
2	Rogowiec (vicinity of Belchatów) N 51°16'40" E 19°16'00"	25	15	163 (36%)	228 (51%)	0	0.182
3	Brzozowe Błota (Bory Tucholskie region) N53°39'50" E 18°08'00"	10	5	117 (26%)	117 (26%)	0	0.145
4	Biała (Bory Tucholskie region) N 53°40'40" E 17°59'20"	80- 100	15	204 (46%)	291 (65%)	3	0.214
5	Kielpiński Most (Bory Tucholskie region) N 53°19'20" E 17°59'20"	2	2	62 (14%)	62 (14%)	0	0.139
6	Ustroń (Bory Tucholskie region) N 53°45'30" E 18°01'20"	6	5	159 (36%)	159 (36%)	2	0.180
7	Osie (Bory Tucholskie region) N 53°37'45" E 18°20'55"	40	6	148 (33%)	156 (35%)	0	0.162

produced fragment patterns. In total, 446 scorable fragments were obtained. The details of the PCR protocols and oligonucleotide sequences used are available from the author upon request. All the incubation steps were carried out in a T3 Thermocycler (Biometra). Selective amplification electrophoresis was performed using an automated sequencer ABI 310 (Applied Biosystems) with GeneScan 500 Rox size standard (Applied Biosystems). AFLP patterns were visualized with GeneScan Analysis software 3.1 (Applied Biosystems). The presence/absence of AFLP bands was scored using Genographer software [10].

Data analysis

The proportions of mono- and polymorphic loci were calculated to check the overall genetic diversity. Private fragments, unique for the respective populations, were also noted. The genetic diversity level of polymorphic loci was represented by the average gene diversity index, equivalent to the expected

heterozygosity in diploid data [11]. Analysis of molecular variance (AMOVA [12]) was performed to estimate the distribution of genetic diversity within and between populations. These data were obtained using Arlequin 2.00 software [13]. A UPGMA clustering of all the individuals, based on a similarity matrix calculated with Jaccard's coefficient [14], was constructed using NTSYS-pc software [15] to show the relationships between all the populations.

RESULTS AND DISCUSSION

The overall genetic diversity revealed by AFLP analysis was moderate – out of 446 fragments detected, 169 (37.9%) were monomorphic for all populations. Nevertheless, all 59 *P. vernalis* specimens revealed different AFLP phenotypes. Only three populations had single private characteristic fragments (Tab. 1). At the intra-population level, the number of polymorphic loci varied between 14% (in the two-individual population) and 46% (mean value 32.3%) in the normalised number of samples fixed as five per population (the common number for all populations except one composed of only two individuals – no. 5). In the sampling depending on the population size, the number of polymorphic fragments increased in large populations but without changing the general image (Tab. 1). The mean average gene diversity index in populations was 0.170 and varied from 0.139 to 0.204 (Tab. 1). The genetic diversity of populations appears to be only slightly correlated with population size (Tab. 1, Fig. 1); the differences are significant only when the smallest or the largest populations are considered (n° 5 and 4 respectively).

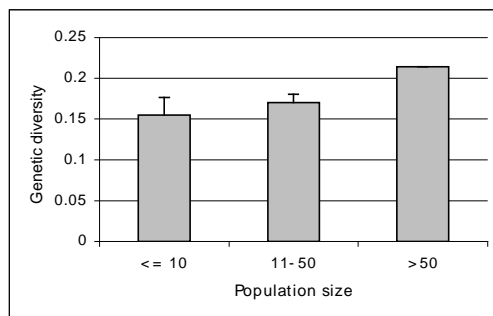


Fig. 1. The relationship between the estimated population size and population molecular diversity inferred from AFLP analysis (average gene diversity). The comparison is based on seven populations (Tab. 1). SD values are shown for the first two groups, the third one is represented by only one population.

The spatial distribution of genetic diversity inferred from the cluster analysis of all the individuals does not show a strong geographical pattern (Fig. 2). Most populations are grouped in population-specific clusters, but three populations from the Bory Tucholskie region (n° 3, 4, 7) are intermixed. Only one population

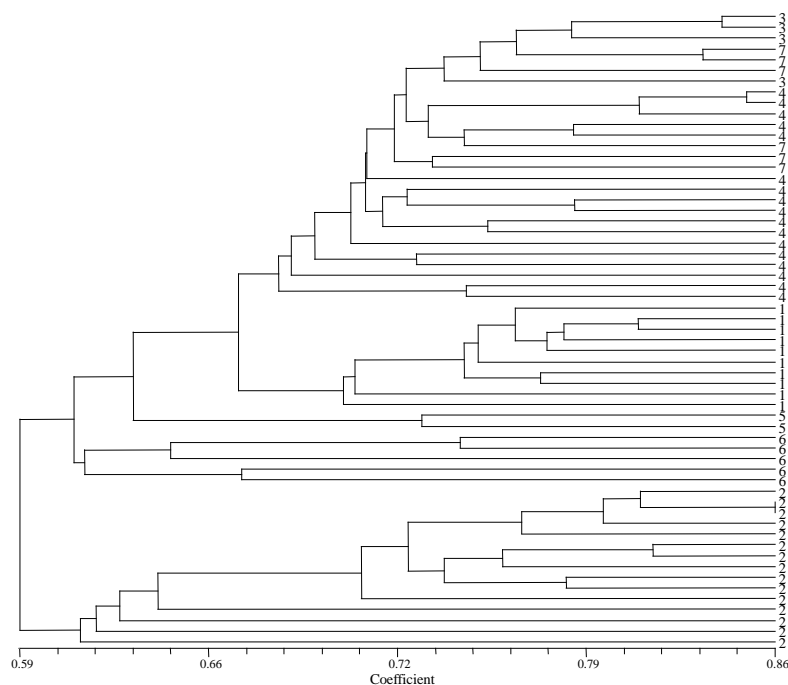


Fig. 2. UPGMA clustering of 58 individuals of *P. vernalis* from 7 populations (numbers 1-7 refer to the population numbers as given in Tab. 1, using 446 AFLP fragments, computed with a similarity matrix based on Jaccard's coefficient).

(n° 2 – Central Poland) forms a separate group; surprisingly, population n° 1 (Kielce, Southern Poland), which is spatially the most isolated, is grouped within the big cluster of populations from Bory Tucholskie (Northern Poland). Noteworthy, the UPGMA tree shows a predominant within-population diversity rather than between-population clustering. The AMOVA analysis (Tab. 2) provides strong support for these observations, showing that the main variation component (app. 72%) is contributed to by the within-population level, even though the between-population variation is also significant (the variation between populations in Northern, Central and Southern Poland is not significant – data not shown). Relating the intrapopulation genetic diversity of *P. vernalis* inferred from AFLP analysis with the data from the literature as compared in [8] allows us to make the general statement that the species has an intermediate level of diversity. The significant differences between the smallest and the largest populations confirm that big populations are more likely to maintain a high level of diversity [16]. Nevertheless, even small populations (5-10 individuals) exhibit relatively high diversity index values (compared with their size). This fact, together with the rather low spatial structurization of genetic

diversity at the between-population level (typical for outcrossing and perennial plants [17]) could reflect the recent history of the strong decrease in *P. vernalis* populations, as suggested for some other rare species [e.g. 18, 19]. It could be expected that, with time, the diversity will decrease due to this recent isolation [20], especially as the structure analysis of the large population reveals some substructuring, suggesting a probable predominance of short-distance seed dispersal (data not shown). Far more than fifty per cent of the known localities

Tab. 2. Results of the Analysis of Molecular Variance (AMOVA) for 58 individuals of *P. vernalis* from 7 populations, using 446 AFLP fragments. Levels of significance are based on 1023 random permutations. Fixation index (FST) amounts to 0.2822.

Source of variation	d.f.	Variance component	Percentage of variation	P
Among populations	6	14.90	28.22	<0.001
Within populations	52	37.91	71.78	<0.001

of this species in Poland, and almost all in the neighbouring countries, disappeared within the last 100 years [2, 3, 4], mainly because of the disturbance of habitats resulting from intensive forest exploitation. However, the relationship between population size and diversity should be interpreted with caution, as it can be biased to some extent by annual fluctuations reported for *P. vernalis* [3]. Clearly, very rare large populations (about 100 individuals) have considerably higher genetic diversity and should be protected as the main sources of a species' diversity, but also in all the intermediate populations the diversity level is rather constant and not low. Although the proportion of monomorphic loci is relatively elevated, the diversity within polymorphic loci is considerable. In active management, the exchange of genotypes between populations could probably limit the potential depression in the future. The main effort in protection strategy should doubtlessly be put on preserving the habitats of the remaining populations, as the ecological demands of the plant are very strict and small populations are very susceptible to stochastic events.

As a further step, a detailed analysis of genetic structure will be performed to establish guidelines for *ex situ* seed sample collections. A comparison of the genetic diversity of *P. vernalis* in the Polish lowlands (a strongly disturbed population structure) with that in the Alps (the potential centre of the species' occurrence with abundant and well-connected populations) will provide the background for inferring conclusions concerning the character of the species' diversity in Poland.

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