LOW GENETIC DIVERSITY OF DECLINING VIOLA ULIGINOSA (VIOLACEAE) AT ITS SOUTHERN RANGE LIMITS IN POLAND

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Viola uliginosa (bog violet) is a declining species throughout its range due to – mostly anthropogenic – drying out of the wet habitats it occupies. Using AFLP markers, we aimed to estimate the genetic diversity in Polish populations, that may give an insight into the situation of plant populations facing rapid loss of natural habitats.

Bog violet from several dispersed Polish populations is generally characterized by very low genetic diversity ($H_T = 0.048$), even lower than several other endangered violets; therefore, we suggest that it should preserve at least EN rank in the red lists/red data books. The mean gene diversity within all populations ($H_s$) was much lower than gene diversity ($G_{ST}$) between populations (0.020 versus 0.583, respectively) which supports the prevalence of clonal propagation of the species (mainly by stolons) but may also point to some significance of autogamy in cleisto- and chasmogamous flowers. A high $F_{ST}$ value and the Mantel test for all populations revealed significant isolation by distance. Geographically neighboring pairs of populations formed genetic clusters supported by all (in the case of two closest populations) or most statistical analyses applied. Special attention should be paid to the locus classicus of the species in Rząska, consisting of a small number of individuals, forming a genetically distinct group, revealing very low gene diversity ($H_s = 0.009$) and the longest genetic distance to the remaining populations. Our results can contribute to planning future protection measures for the species at this and other locations. Genetic structure of the studied populations suggests local affinities of populations but does not generally support hypothesized recent continuity of V. uliginosa range along the river valleys of southern Poland; this view may, however, be altered with widening of the scope of studied populations and chosen molecular markers.

Keywords: Viola uliginosa, Poland, wet habitats, genetic variation, AFLP markers, species conservation, locus classicus

INTRODUCTION

Viola uliginosa Besser (bog violet) is an East- and Central-European species of wet terrestrial habitats. Its distribution is restricted to southern and southeastern Fennoscandia in the north, eastern Germany in the west, western Russia in the east and Poland as well as south-western Ukraine (with disjunctive locations in Slovenia and Croatia) in the south (Hegi, 1925; Valentine, 1968; Meusel et al., 1978; Böhm and Stetzka, 2003; Marcussen and Karlsson, 2010; Paul et al., 2014). The species occurs mostly in wet alder woods with Alnus glutinosa and willow woods with Salix spp. and on fens and transition bogs. Nowadays, it can also be found in and along artificial ditches draining its original habitats. Due to the falling ground water level at many locations, resulting from both artificial drain-
age and the general climatic trend observed for at least several years, the habitats of *V. uliginosa* are shrinking. As a consequence, the size and number of its populations show a trend of drastic decline. In most countries where populations persist, it is regarded as a rare and endangered element of the flora (Kirschner and Skalicky, 1990; Paul et al., 2014; Matulevičiūtė, 2015); it was also included into the red list of plants for the whole Baltic region (Ingelög et al., 1993). In Poland gradual disappearance of the suitable habitats and loss of the most of the historical sites has been observed (only populations in the south-eastern part of the country have been confirmed in the last decades; Paul et al., 2014) and consequently, since 2004 *V. uliginosa* has been a law-protected species. It has also been included in the Polish red list as a critically endangered (CR) species (Zarzycki and Szeląg, 2006) and in the red data book (CR rank, subsequently lowered to VU – see Discussion – Baryla and Kuta, 2001; Paul et al., 2014). Active species conservation has already been attempted via *in vitro* culture as part of *ex situ* conservation. Although micropropagation via direct and indirect organogenesis was successful, 36–75% of regenerated plants (depending on experiment) were tetraploid, in spite of being derived from a diploid (2n = 20) tissue, due to somaclonal variation. Regenerated tetraploids producing a higher than diploids amount of cyclotides (plant defensive peptides) have rather pharmaceutical value and do not provide an appropriate material for introduction into the field due to their uncertain genetic stability (Slazak et al., 2015a, 2015b).

*V. uliginosa* is a rhizomatous, acaulescent, perennial violet. It has chasmogamous (CH) flowers and its lack of cleistogamous (CL) flowers had been assumed before (Becker, 1925; Zablocki, 1947; Valentine, 1968; Kuta, 1978; Marcussen and Karlsson, 2010). However, the presence of cleistogamy has recently been recorded for the first time in one of the Polish populations (Nowa Dęba, NDB; Malobecki et al., 2016). Genetic studies on the *locus classicus* population (Cieślak et al., 2006) revealed its very low genetic diversity, thus the question arose, whether it is characteristic of the species over a wider range or only of that very limited declining population.

Hybridization, another risk factor for the species, can be regarded as rather low. Taxonomic position of *V. uliginosa* has long been uncertain (Tzvelev, 2002). It was previously placed in the monotypic subsection *Repentes* (Kupffer) Juz. of section *Viola* L. (Kupffer, 1903; Becker, 1925; Valentine, 1968; Nikitin, 1998) or even the separate section *Icmasion* Juz. ex Tzvel. (Tzvelev, 2000). Recent studies by means of a multigene multispecies coalescent analysis (Marcussen et al., 2015; Malobecki et al., 2016) have indicated *V. uligino-

**MATERIAL AND METHODS**

**FIELD STUDY**

Five spatially representative populations (all known at the time of the field research) were sampled in the years 2002–2008 across the range of *V. uliginosa* in Poland (Fig. 1; Table 1). Up to three fresh...
leaves per individual (rosette) were enclosed in a blotting paper filter and placed in the field in plastic zip-lock bags filled with dried silica gel. To avoid multiple sampling of existing clones, the leaves were collected from rosettes at least 10 m distant from each other or, if the physical size of the population was too small, at least from the rosettes clearly isolated (i.e., separated by a stream bed, growing on separate tussocks, etc.). The sampling pattern applied was aimed at possibly uniform coverage of all representative fragments of a population (for separate, supplementary rounds of sampling in RZA and NDB, separate subpopulations were chosen). If the size of the population was sufficient, a voucher specimen was collected and deposited in the herbarium KRAM.

LABORATORY ANALYSES

Total genomic DNA was isolated from ca. 20 mg of leaf tissue (dry mass) per sample using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA quality and concentration were checked on 1% agarose gel. The genetic analysis was carried out using Amplified Fragment Length Polymorphism (AFLP) method (Vos et al., 1995; modified as described by Ronikier et al., 2008).

Genomic DNA was digested using Eco RI and Mse I restriction enzymes (New England Biolabs, Ipswich, USA) and subsequently adapters

TABLE 1. Basic data on sampled Viola uliginosa populations (in geographic longitude order) and results of multilocus statistics. $N$ – number of individuals analyzed, $N_{hap}$ – number of haplotypes, $H_{hap}$ – estimate of clone-corrected Nei gene diversity, $I_{hap}$ – estimate of clone-corrected Shannon index, $I_A$ – index of multilocus association after Brown et al. (1980), $r_d$ – index of multilocus association after Burt et al. (1999). In brackets at $H_{hap}$ and $I_{hap}$ ±SD values are given. All values of multilocus indices are significant at $P = 0.05$.

<table>
<thead>
<tr>
<th>No.</th>
<th>Acronym</th>
<th>Station</th>
<th>Coordinates</th>
<th>Sampling year(s)</th>
<th>$N$</th>
<th>$N_{hap}$</th>
<th>$N_{hap}/N$</th>
<th>$H_{hap}$</th>
<th>$I_{hap}$</th>
<th>$I_A$</th>
<th>$r_d$</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RZA</td>
<td>Rząska (W Kraków)</td>
<td>50°05’N 19°51’E</td>
<td>2002 2005 2008</td>
<td>20</td>
<td>3</td>
<td>0.15</td>
<td>0.021</td>
<td>0.30</td>
<td>2.76</td>
<td>0.59</td>
<td>locus classicus</td>
</tr>
<tr>
<td></td>
<td>KAM</td>
<td>Kamionki (SW Nowy Majdan Królewski)</td>
<td>50°22’N 21°43’E</td>
<td>2003</td>
<td>14</td>
<td>8</td>
<td>0.57</td>
<td>0.034</td>
<td>0.055</td>
<td>1.43</td>
<td>0.09</td>
<td>station from the paper by Cieślak et al., 2004</td>
</tr>
<tr>
<td>3</td>
<td>NDB</td>
<td>Nowa Dęba</td>
<td>50°25’N 21°47’E</td>
<td>2005 2006</td>
<td>22</td>
<td>10</td>
<td>0.45</td>
<td>0.049</td>
<td>0.075</td>
<td>3.26</td>
<td>0.18</td>
<td>station from the paper by Malobocki et al., 2016</td>
</tr>
<tr>
<td>4</td>
<td>GIE</td>
<td>Giełnia (S Zaklików)</td>
<td>50°43’N 22°05’E</td>
<td>2008</td>
<td>12</td>
<td>2</td>
<td>0.17</td>
<td>0.012</td>
<td>0.016</td>
<td>2.00</td>
<td>1.00</td>
<td>station from the paper by Krawczyk et al., 2008</td>
</tr>
<tr>
<td>5</td>
<td>SIE</td>
<td>Rudka (N Sieniawa)</td>
<td>50°14’N 22°36’E</td>
<td>2004</td>
<td>15</td>
<td>1</td>
<td>0.07</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>station from the paper by Krawczyk et al., 2008</td>
</tr>
</tbody>
</table>
were ligated by T4 ligase (Roche, Basel, Switzerland). Restriction/ligation products were amplified using preselective starters Eco-A and Mse-C. For selective amplification, 14 primer sets were tested in the pilot study for an optimal number of bands, repeatability, unequivocal readout and analysis. Three primer pairs were subsequently chosen and used in the analysis: Eco-AAG – Mse-CGC, Eco-AAG – Mse-CGT and Eco-ATT – Mse-CAT (primers synthesized by Applied Biosystems, Waltham, USA). Eco- primers were fluorescent-labeled (6-FAM). Electrophoresis of AFLP markers was conducted in POP-4 polymer with GeneScan-500 fragment size standard (Rox 500) using an ABI Prism 3100-Avant sequencer (Applied Biosystems). AFLP analysis of all samples was conducted in a single laboratory campaign, to avoid instrumental and technical differentiation among readings. Seven percent of samples were used as duplicates to assess the genotyping error and repeatability of data. Tests revealed 82% mean repeatability in particular starter sets. Only the repeatable bins were considered in further analyses.

STATISTICAL ANALYSES
Genetic diversity was estimated by calculating related parameters at the level of populations and of the whole study area. The number (P) and proportion (%pop) of polymorphic markers, the number of private markers (i.e., those occurring only within a particular group, Nₚₚ), the number of discriminating markers (i.e., those fixed for particular groups, Nₖ), Nei’s gene diversity (Hₑ; Nei, 1973) and Shannon’s information index (Iₑ; Lewontin, 1972) were calculated at the population level. Study area level parameters included total gene diversity (Hₛₑ), within-population mean gene diversity (Hₛₑ; Nei, 1973), among-population gene diversity (Gₛₑ), and estimated gene flow (Nₑₑ; Slatkin and Barton, 1989). These parameters were calculated using FAMD v. 1.25 (Schlüter and Harris, 2006) and POPGENE v. 1.31 (Yeh et al., 1999). Clonal correction of gene diversity was done by haplotype identification in AFLPdat (Ehrich, 2006) with the maximum number of differences set empirically to two, based on results of a separate analysis (Paul et al., unpublished data). Clone-corrected Nei’s gene diversity (Hₛₑₑ) and Shannon’s information index (Iₛₑₑ) were calculated in POPGENE v. 1.31.

For relationships between individuals and populations, a NeighborNet was constructed in SplitsTree v. 4.6 (Huson and Bryant, 2006) based on a matrix of Nei & Li coefficients (Nei and Li, 1979). Split support was calculated by bootstrapping with 1000 replicates. Principal coordinate analysis (PCoA) was performed with Nei & Li distance coefficients in FAMD v. 1.25.

Analysis of population genetic structure was carried out based on Bayesian inference cluster-

ing in STRUCTURE v. 2.3 (Pritchard et al., 2000; Falush et al., 2007) assuming recessive allele model for dominant markers, admixture model and independent allele frequencies between clusters. For each K value from 2 to 10, ten independent runs were performed with a burn-in of 200,000 followed by 1,000,000 Markov Chain Monte Carlo replicates. To determine the optimal K value, the estimated mean logarithmic likelihood of K values and AK values were calculated (Evanno et al., 2005) using Structure Harvester v. 0.6 (Earl and VonHoldt, 2012). Summation of population structure results was performed in CLUMPAK (Kopelman et al., 2015) using LargeKGreedy search method and 2000 random input repeats.

A pairwise fixation index (Fₛₑ) distance matrix, pairwise estimated gene flow (Nₑₑₑ) between populations and three-level hierarchical analysis of molecular variance (AMOVA) was performed in ARLEQUIN v. 3.5 (Excoffier et al., 2005). AMOVA was performed on all individuals and population groups suggested by STRUCTURE using pairwise difference distance matrix at P = 0.05. To evaluate the correlation between populations’ Fₛₑₑ distance and geographical distances, the Mantel test (1023 permutations) was performed in ARLEQUIN v. 3.5.

Multilocus analysis was performed by measuring the observed value of the index of association (Iₑₑ), which is a summary statistic of association between loci (Brown et al., 1980) and the rₑₑ association index, which is independent of the analyzed loci number. Calculations were performed using MultiLocus software v. 1.2 (Agapow and Burt, 2001).

RESULTS
AFLP analysis yielded a total of 129 bands, 41 of which (31.8%) were polymorphic in the total data set with a mean of 9.6% across all populations. The highest percentage of polymorphic bands was found in NDB (17.1%) and KAM (13.2%) populations, while the lowest (5.4%) in GIE population. Private bands were present in all the populations except RZA. A discriminating marker fixed for all individuals within a population was recorded only in GIE. Nei’s gene diversity (Hₑ) was highest in NDB and KAM populations (0.036 and 0.032, respectively) and in the remaining ones it was three to four times lower (with minimum in RZA: 0.009). The mean diversity within all populations (Hₛₑ) was low (0.020), whereas diversity among populations (Gₛₑₑ) was high (0.583). The estimated gene flow between populations (Nₑₑₑ) was 0.358 (Table 2).

The principal coordinate analysis (PCoA) revealed three distinct genetic groups. One of them corresponded to a single population (RZA). Each of
the remaining two groups consisted of two populations (KAM and NDB versus GIE and SIE) with the KAM and NDB group showing the highest variance across both components (Fig. 2a). The pairwise $F_{ST}$ distance between populations was the highest between GIE and RZA populations and between GIE and SIE populations (both 0.823). The shortest distance was estimated between KAM and NDB (0.099). Accordingly, the pairwise estimated gene flow ($N_m$) was highest between KAM and NDB populations (4.903) and lowest between GIE and RZA (0.124) (Table 3).

In the NeighborNet analysis a group formed by KAM and NDB populations was weakly supported (bootstrap below 50%), while the support for groups splitting other populations (SIE, RZA and GIE) was significantly higher (Fig. 2b).

The optimal number of groups in the Bayesian STRUCTURE analysis was established as $K=3$ ($\Delta K = 14.98$). The results for the highest number of runs (5 out of 10) showed a clear division of the populations into three groups corresponding to those observed in PCoA. The first consisted of GIE and SIE populations, the second comprised KAM

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### Table 2. Parameters of genetic diversity in Polish populations of *Viola uliginosa*. N – no. of individuals used in genetic analyses; P – no. of polymorphic markers; %poly – proportion of polymorphic markers; $N_{prt}$ – no. of private markers (present only in a given population); $N_d$ – no. of discriminating markers (present in all individuals of a given population, but absent in other populations); $H_j$ – Nei’s (1973) gene diversity; $I$ – Shannon’s information index; $H_T$ – Nei’s (1973) total gene diversity; $H_S$ – Nei’s (1973) mean gene diversity within populations; $G_{ST}$ – Nei’s (1973) gene diversity among populations; $N_m$ – estimated gene flow. In brackets at $H_j$, $I$, $H_T$ and $H_S$ ±SD values are given. For population acronyms see Table 1.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>P (%)poly</th>
<th>$N_{prt}$</th>
<th>$N_d$</th>
<th>$H_j$ (±SD)</th>
<th>$I$ (±SD)</th>
<th>$H_T$ (±SD)</th>
<th>$H_S$ (±SD)</th>
<th>$G_{ST}$</th>
<th>$N_m$ (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. uliginosa</em></td>
<td>83</td>
<td>41 (31.8%)</td>
<td>0</td>
<td>0</td>
<td>0.048 (±0.114)</td>
<td>0.081 (±0.171)</td>
<td>0.583</td>
<td>0.358</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RZA</td>
<td>20</td>
<td>8 (6.2%)</td>
<td>0</td>
<td>0</td>
<td>0.009 (±0.047)</td>
<td>0.017 (±0.075)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KAM</td>
<td>14</td>
<td>17 (13.2%)</td>
<td>5</td>
<td>0</td>
<td>0.032 (±0.095)</td>
<td>0.053 (±0.147)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDB</td>
<td>22</td>
<td>22 (17.1%)</td>
<td>5</td>
<td>0</td>
<td>0.036 (±0.099)</td>
<td>0.060 (±0.151)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIE</td>
<td>12</td>
<td>7 (5.4%)</td>
<td>2</td>
<td>1</td>
<td>0.012 (±0.055)</td>
<td>0.020 (±0.088)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIE</td>
<td>15</td>
<td>8 (6.2%)</td>
<td>3</td>
<td>0</td>
<td>0.011 (±0.050)</td>
<td>0.020 (±0.084)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Matrix of pairwise $F_{ST}$ distances (below diagonal) and estimated gene flow ($N_m$) values (above diagonal) between Polish populations of *Viola uliginosa*. All $F_{ST}$ values are statistically significant at $P = 0.05$. For population acronyms see Table 1.

<table>
<thead>
<tr>
<th></th>
<th>RZA</th>
<th>KAM</th>
<th>NDB</th>
<th>GIE</th>
<th>SIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RZA</td>
<td>0.706</td>
<td>0.617</td>
<td>0.823</td>
<td>0.128</td>
<td></td>
</tr>
<tr>
<td>KAM</td>
<td>0.229</td>
<td>4.903</td>
<td>0.612</td>
<td>0.357</td>
<td></td>
</tr>
<tr>
<td>NDB</td>
<td>0.341</td>
<td>0.099</td>
<td>0.584</td>
<td>0.452</td>
<td></td>
</tr>
<tr>
<td>GIE</td>
<td>0.124</td>
<td>0.342</td>
<td>0.387</td>
<td>0.193</td>
<td></td>
</tr>
<tr>
<td>SIE</td>
<td>0.823</td>
<td>0.607</td>
<td>0.549</td>
<td>0.750</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Intra- and interpopulational genetic diversity of 83 individuals of Viola uliginosa from five populations based on AFLP results. For population acronyms see Table 1. (a) – Principal Coordinate Analysis (PCoA) (first two axes). Percentage of total variation for each component is given in brackets. (b) – NeighborNet analysis. Bootstrap values shown (>50) were calculated on 1,000 replicates.
and NDB, while the RZA population formed a separate, third group. Some individuals from GIE and NDB populations showed some admixture of RZA gene pool (Suppl. Fig. 1a). The minority of runs (4 out of 10) showed division into two groups only (the third constituting only a negligible admixture in one of NDB individuals), with KAM and NDB forming the first and the remaining three populations the second group (Suppl. Fig. 1b). The remaining run again suggested division into two groups, but in this case the first one consisted of RZA only, while the remaining populations formed the second one (Suppl. Fig. 1c).

The analysis of molecular variance (AMOVA) done for the STRUCTURE-based triple division, revealed only slightly higher percentage of variation within populations (37.50%) than among the three population groups (34.95%), both being much lower than that among all populations (59.99%). The highest variation index was observed for the variance among populations relative to the total variance ($F_{ST} = 0.625$), while the variation among population groups ($F_{CT} = 0.350$) was on the verge of statistical significance ($P = 0.057$). The AMOVA repeated for the two-group division showed the lowest percentage of variation among groups (17.70%), the highest variation among populations ($F_{ST} = 0.648$) and insignificant variation among population groups ($P = 0.102$) (Table 4). The Mantel test showed a significant correlation between $F_{ST}$ and geographical distances ($P = 0.034$) with the correlation coefficient at 0.738 and determination of genetic distance by geographical distance equaling 0.545%.

The number of genotypes identified in V. uliginosa populations (with tolerance of clone diversity set to two markers, see above) ranged from one in SIE population to 10 in NDB. Clone corrected gene diversity was highest in NDB ($H_{hap} = 0.049$; $I_{hap} = 0.075$), and lowest in GIE population ($H_{hap} = 0.012$; $I_{hap} = 0.016$) (these values were not calculated for SIE, as having only one effective genotype, Table 1). Multilocus analysis performed on four populations with a sufficient (>1) number of genotypes showed the highest value of association index ($I_A$) in the NDB (3.26), and the lowest in the KAM population (1.43). The $r_d$ index, however, was highest in GIE and RZA populations.

### Table 4. Result parameters of the AMOVA performed on 5 populations of Viola uliginosa grouped according to STRUCTURE results (three-group and two-group variant). df – degrees of freedom, $P$ – significance level (if $F$ values marked by *, then $P < 0.001$).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
<th>Fixation indices</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V. uliginosa: all populations studied</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among populations</td>
<td>4</td>
<td>147.11</td>
<td>2.157</td>
<td>59.99</td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>78</td>
<td>112.19</td>
<td>1.438</td>
<td>40.01</td>
<td>$F_{ST} = 0.600^*$</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>259.30</td>
<td>3.595</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td><strong>V. uliginosa: three-group division</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>2</td>
<td>112.07</td>
<td>1.341</td>
<td>34.95</td>
<td>$F_{CT} = 0.350$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$P = 0.057$</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>2</td>
<td>35.05</td>
<td>1.057</td>
<td>27.55</td>
<td>$F_{SC} = 0.423^*$</td>
</tr>
<tr>
<td>Within populations</td>
<td>78</td>
<td>112.19</td>
<td>1.438</td>
<td>37.50</td>
<td>$F_{ST} = 0.625^*$</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>259.30</td>
<td>3.836</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td><strong>V. uliginosa: two-group division</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>1</td>
<td>59.21</td>
<td>0.657</td>
<td>17.70</td>
<td>$F_{CT} = 0.177$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$P = 0.102$</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>3</td>
<td>87.42</td>
<td>1.749</td>
<td>47.12</td>
<td>$F_{SC} = 0.573^*$</td>
</tr>
<tr>
<td>Within populations</td>
<td>78</td>
<td>101.83</td>
<td>1.305</td>
<td>35.18</td>
<td>$F_{ST} = 0.648^*$</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>248.45</td>
<td>3.711</td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>
Total gene diversity ($H_o$) of Viola uliginosa in the analyzed populations was relatively very low (below 0.05; cf. e.g., a review by Reisch and Bernhardt-Römermann, 2014), comparable to V. graji, a rare, endemic Pacific island species (Hirai et al., 2012), and lower than in other endangered (V. pumila, V. stagnina, V. elatior; Eckstein et al., 2006; Buldrini et al., 2013) as well as common violets (V. riviniana, V. striata, V. grahamii, V. tricolor; Cortes-Palomec et al., 2006; Słomka et al., 2011; Kuta et al., 2014; Migdałek, 2015). The mean gene diversity within all populations ($H_s$), being much lower than gene diversity among populations ($G_{ST}$) (0.020 versus 0.583, respectively), strongly supported domination of clonal reproduction and propagation of the species. As shown by Nybom and Bartish (2000) and Nybom (2004), the mean among-population variability for obligatorily selfing species is evidently higher ($G_{ST} = 0.70$ and 0.59, respectively) than that for outcrossing species ($G_{ST} = 0.23$ and 0.22). The other $G_{ST}$-enlarging life history trait accounted for by Nybom (2004) – that of being an annual plant – can be excluded in the case of V. uliginosa. Moreover, this factor was listed as not influencing inter-population diversity in AFLP by Reisch and Bernhardt-Römermann (2014), who pointed instead at another one positively correlated with $\phi_{ST}$: rarity. Although the present V. uliginosa status would promote this one, its influence is also not clear, as the rarity of our study species may likely be a relatively recent (in the microevolutionary timescale of genetic difference expression) effect of habitat shrinking.

In several Viola species with mixed (self-outcrossing) mating systems (e.g., V. cazorlenensis, V. calaminaria, V. pubescens, V. riviniana, V. reichenbachiana, V. striata, V. grahamii, V. tricolor) differentiation among populations is weaker because it is balanced by the existing gene flow (Auge et al., 2001; Culley and Wolfe, 2001; Cortes-Palomec et al., 2006; Bizoux et al., 2008; Słomka et al., 2011; Kuta et al., 2014; Cánovas et al., 2015; Migdałek, 2015). The Mantel test performed on V. uliginosa populations revealed a significant correlation between genetic and geographical distances, showing that gene flow vectors (pollination and diaspore dispersal) are weak even on such relatively short distances. This, together with the high among-population population and low intra-population variation levels (AMOVA results), seems to support the importance of clonality in the species reproduction.

The clonality of V. uliginosa results from vegetative propagation via stolons rather than from self-pollination by CL flowers. In each of the studied populations, significant influence of clonality on genetic diversity has also been detected. Although non-seasonal cleistogamy based on simultaneous presence of chasmogamous (CH) open, self- and cross-pollinated flowers and CL, closed, obligatorily self-pollinated flowers, has recently been detected in the NDB population (Malobecki et al., 2016), so far it has not been observed in other V. uliginosa populations. It has been demonstrated that cleistogamy might influence the genetic structure of violets’ populations. Genetic variation (measured by values of $F_{ST}$ or $\Theta$ indices) in Viola pubescens developing both CH and CL flowers was significantly higher (Culley and Wolfe, 2001) than in V. pedunculata with only CH flowers (Culley and Stokes, 2012). However, in V. striata and V. grahamii high population genetic diversity was maintained, despite very high frequency of CL flowers and vegetative reproduction (Cortes-Palomec et al., 2006). It may be due to a varied fitness of cleistogamosly-derived progeny, which could be lower, higher or comparable to the chasmogamous flower-generated offspring, depending on the species (Berg and Redbo-Torstensson, 1999; Eckstein and Otte, 2005).

Divergent results obtained in multilocus analysis (highest to lowest values of $I_A$ association index order: NDB $\rightarrow$ RZA $\rightarrow$ GIE $\rightarrow$ KAM vs. GIE $\rightarrow$ RZA $\rightarrow$ NDB $\rightarrow$ KAM in the case of the $r_d$ index: Table 1) may reflect the overwhelming effect of the assayed samples number ($N$) on the $I_A$ values, analogically to the latter index sensitivity to the analyzed loci number (Agapow and Burt, 2001). It appears that $N$ and $I_A$ are in fact noticeably correlated here, opposite to $N$ and $r_d$ (R-squared $= 0.79$ for $N$ against $I_A$ vs. 0.19 for $N$ against $r_d$ and correlation coefficient 0.89 vs. -0.44, respectively). This suggests that the $r_d$ index is a more robust base for further consideration in this case. As the indices of multi-locus allele distribution disequilibrium are generally considered correlates of the genetic uniformity of the studied population individuals, the obtained $r_d$ index values suggest that the diversity-limiting phenomena as clonality or selfing play a more significant role in smaller (as GIE and RZA) than larger (as NDB and KAM) populations. Considering that the CL flowers have been so far observed only in NDB, having a relatively low $r_d$ index, we may conclude that obligatory self-pollination plays only a minor role, if any, in maintaining the low genetic diversity of V. uliginosa, clonality being the major factor here.

The limited scope of the present study precludes more general discussion on the range-wide genetic variability of Viola uliginosa, including the question whether the Polish populations – forming part of the southern border of continuous range of
the species (transgressed to the south only by isolated populations in Slovenia and Croatia – Meusel et al., 1978) – do or do not support the classical view (cf. e.g., Eckert et al., 2008) that towards the outer parts of the range inter-population diversity increases at the cost of the intra-population one. We intend to address these in the planned wider-scope study, covering a larger part of the known species’ range.

The geographic distribution of the past and extant known *Viola uliginosa* populations suggests, that they may have formed in the recent past an almost continuous range along the latitudinally oriented system of big river valleys and basins of southern Poland (possibly extending further across Central Europe). Although the presence of distinct genetic groups correlated with spatial isolation among the studied populations (see results of the Mantel test as well as PCoA and NeighborNet analyses compared to the geographic distribution; Figs. 1 and 2) seems not to support this assumption, the impact of the clonal reproduction system may mask possible traces of the past connections. Unfortunately, populations still known in the 1st half of the 20th century (Schube, 1903; Gruhl, 1929; Nowak and Nowak, 2005) from the SW part of the country (Silesia) have not been preserved to date (A. Nowak, M. Nobis, W Paul, unpublished data), precluding a comprehensive testing of this hypothesis. Nevertheless, a good example supporting a scenario of past continuity at least at a more local scale is provided by pairs of neighboring populations grouped by clustering analyses (KAM–NDB and GIE–SIE). Especially the two former geographic close populations are characterized by a high genetic similarity (the pairwise $F_{ST}$ distance = 0.099), probably suggesting a recent disruption of a formerly wider system of mostly clonally persisting populations (although some presence of a recent effective gene flow at a local scale is also a possible factor).

The *locus classicus* population of *V. uliginosa* in Rząska (RZA) should be considered a special protection area. RZA formed a distinct group in PCoA and NeighborNet analyses indicating the longest genetic distance in relation to the remaining populations. Pairwise $F_{ST}$ index among populations also substantiated high genetic differentiation of the RZA population, especially in relation to GIE and SIE (both 0.823). Additionally, it revealed extremely low gene diversity ($H_t = 0.009$), in accordance with the results of Cieśliak et al. (2006) who showed similarity index value close to 1.0 for individuals from this population.

After discovering fourteen large populations in southern Poland not known before, Krawczyk et al. (2008) suggested to decrease the species national threat category from CR to VU (that was acknowledged by Paul et al., 2014). Our present results on genetic diversity and population structure of the species do not support such an essential change. Newly discovered populations reported by Krawczyk et al. (2008) are evidently not the effect of the contemporary species expansion since *V. uliginosa* genetic structure indicates clonality (present results) as the dominant way of propagation with seed dispersal playing an insignificant role (even if myrmecochory is involved – see Culver and Beattie, 1980). Thus, those populations would also represent remains of past population resources that must have not been recorded before, probably due to the short flowering period of *V. uliginosa* in hard-accessible areas (high water level in the spring), summer dominance of taller plants or just simply due to the lack of botanical survey in these areas. GIE and SIE populations, being among those newly discovered, are genetically almost as homogeneous as the previously studied declining RZA population (Cieśliak et al., 2006).

Although the presence of numerous individuals was observed at some locations (e.g., KAM, NDB) in drainage ditches dehydrating original habitats of the species, this does not necessarily mean that it presents an ecological plasticity that may ensure long-term survival. When the drained original habitat, being a source of the diaspores/clonal dispersal fragments, disappears, there will be no possibility to re-colonize in the case of the ditch cleaning/renovation, usually taking place every several years. Vulnerability of the species’ habitat taken together with the low general genetic diversity ($H_T = 0.048$) of the country’s metapopulation is in favor of categorization of *V. uliginosa* as at least an endangered (EN) species in Poland.

Due to the high genetic uniformity of the individuals, a random, spatially-dispersed (seed or tissue) sampling from 10-20% of the rosettes per population (estimated clone-corrected genotypes number, see Table 1, may be helpful in determining the necessary sampling size) should be sufficient to preserve the representative part of its present genetic diversity via *ex situ* conservation and/or, if necessary, by *in vitro* plant regeneration with subsequent acclimatization (see e.g., Slazak et al., 2015b with caveats as in Introduction).

**CONCLUSION**

Our study shows a very limited genetic diversity of *Viola uliginosa* at the regional scale, most probably derived from the dominant clonal way of dispersal. This is further supported by a conspicuous isolation-by-distance pattern revealed by genetic vs. geographic distance Mantel test. Considering low genetic diversity of the species and its very narrow
ecological spectrum (its phytosociological faithfulness to few biotope types, considered endangered at the national and continent-wide scales), V. uliginosa, in our opinion, should remain at least at EN national threat category and its locus classicus (RZa population) should be given the highest protection priority.

AUTHORS’ CONTRIBUTIONS

WP conceived, designed and coordinated the study. WP and EC conducted field studies. WP, EC and MR performed laboratory analyses. WP, EC, MR, GM and JŻ analyzed the data statistically. All authors contributed to writing and editing the manuscript, led by WP.

All authors have read and approved the final manuscript. The authors declare no conflict of interest.

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