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Low Genetic Diversity of Declining *Viola uliginosa* (Violaceae) at its Southern Range Limits in Poland

Wojciech Paul[1*], Elżbieta Cieślak[1], Michał Ronikier[1], Grzegorz Migdałek[2], Aneta Słomka[3] and Justyna Żabicka[3]

[1]*W. Szafer Institute of Botany, Polish Academy of Sciences, Lubicz 46, 31-512 Cracow, Poland*

[2]*Department of Plant Physiology, Pedagogical University of Cracow, Podchorążych 2, 30-084 Cracow, Poland*

[3]*Department of Plant Cytology and Embryology, Jagiellonian University, Gronostajowa 9, 30-387 Cracow, 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_j = 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-

* Corresponding author, email: w.paul@botany.pl

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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 Skalický, 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 – Baryła 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; Zabłocki, 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; Małobęcki 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; Małobęcki et al., 2016) have indicated *V. uliginosa* as a strongly supported lineage within subsect. *Rostratae* Kupffer of section *Viola* which was also supported by the presence cleistogamy (common in *Rostratae*) recorded recently (Małobęcki et al., 2016). Close relationship of *V. uliginosa* with the subsect. *Rostratae* is also supported by its ability to form hybrids with *V. canina*, *V. reichenbachiana*, *V. riviniana* and *V. stagnina* of the sect. *Rostratae* but not with species of other subsections of *Viola* section (Kuta, 1978; Marcussen and Karlsson, 2010). However, hybridization does not seem to be the factor promoting extinction of *V. uliginosa* (for description of such mechanisms see, e.g., Stace, 1975; Wolf et al., 2001; Gómez et al., 2015) because hybrids are rarely formed in natural habitats and they are almost sterile. The first generation hybrids of *V. uliginosa* × *V. riviniana* (= *V.* × *eriksoniana* W. Becker) found in *locus classicus* in Poland were sterile, forming only 1.3% of viable pollen and deformed capsules without seeds (Kuta, 1978). For a long time, exclusively pure species of *V. riviniana* and *V. uliginosa*, without traces of introgression, were observed in the *locus classicus* (Cieślak et al., 2006). In 2008, the presence of only two individuals of *V. uliginosa* × *riviniana* hybrid was confirmed both morphologically and by AFLP analysis (W. Paul, E. Cieślak, unpublished data). All this points to the rarity of the hybridization process and that it is not likely to influence the genetic stability and identity of the pure species.

Due to its ecological properties and the observed trends of populations' decline in the number and size, *V. uliginosa* can be considered a very representative example of a species strongly endangered by contemporary (destructive) anthropogenic influence on habitats together with global climatic trends changing properties of local climatic niches for species.

In this paper we aimed at estimation of within- and among-population genetic diversity of *Viola uliginosa* at the southern limits of its core distribution range (Poland) and to contribute to the knowledge of current genetic properties of rapidly declining species attached to vulnerable European wet habitats. Our results also provide an important rationale when considering future passive and active protection measures for this declining species in Poland and in the neighboring countries.

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

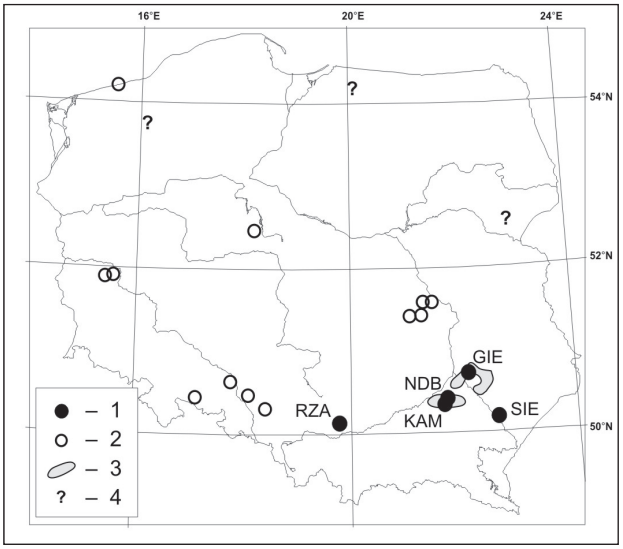

Fig. 1. Situation of the sampled *Viola uliginosa* populations in Poland against the background of the species' known historical and present distribution in the country (after Paul et al., 2014, modified). Legend: 1 – sampled populations, 2 – historical locations, 3 – areas of numerous extant populations, 4 – doubtful locations.

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 $P = 0.05$.

No.	Acronym	Station	Coordinates	Sampling year(s)	N	N_{hap}	N_{hap}/N	H_{hap}	I_{hap}	I_A	r_d	Remarks
1	RZA	Rząska (W Kraków)	50°05'N 19°51'E	2002 2005 2008	20	3	0.15	0.021 (±0.094)	0.030 (±0.135)	2.76	0.59	*locus classicus*
2	KAM	Kamionki (SW Nowy Majdan Królewski)	50°22'N 21°43'E	2003	14	8	0.57	0.034 (±0.099)	0.055 (±0.153)	1.43	0.09	station from the paper by Cieślak et al., 2004
3	NDB	Nowa Dęba	50°25'N 21°47'E	2005 2006	22	10	0.45	0.049 (±0.127)	0.075 (±0.188)	3.26	0.18	station from the paper by Małobęcki et al., 2016
4	GIE	Gielnia (S Zaklików)	50°43'N 22°08'E	2008	12	2	0.17	0.012 (±0.076)	0.016 (±0.104)	2.00	1.00	station from the paper by Krawczyk et al., 2008
5	SIE	Rudka (N Sieniawa)	50°14'N 22°36'E	2004	15	1	0.07	–	–	–	–	station from the paper by Krawczyk et al., 2008

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*-AGG – *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 ($\%_{poly}$) of polymorphic markers, the number of private markers (i.e., those occurring only within a particular group, N_{prt}), the number of discriminating markers (i.e., those fixed for particular groups, N_d), Nei's gene diversity (H_j; Nei, 1973) and Shannon's information index (I; Lewontin, 1972) were calculated at the population level. Study area level parameters included total gene diversity (H_T), within-population mean gene diversity (H_S; Nei, 1973), among-population gene diversity (G_{ST}) and estimated gene flow (N_m; 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 (H_{hap}) and Shannon's information index (I_{hap}) 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 clustering 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 ΔK 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 (F_{ST}) distance matrix, pairwise estimated gene flow (N_m) 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' F_{ST} 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 (I_A), which is a summary statistic of association between loci (Brown et al., 1980) and the r_d 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 (H_j) 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 (H_S) was low (0.020), whereas diversity among populations (G_{ST}) was high (0.583). The estimated gene flow between populations (N_m) 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

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.

Population	N	P ($\%_{poly}$)	N_{prt}	N_d	H_j	I	H_T	H_S	G_{ST}	N_m
V. uliginosa							0.048 (±0.013)	0.020 (±0.002)	0.583	0.358
RZA	20	8 (6.2%)	0	0	0.009 (±0.047)	0.017 (±0.075)				
KAM	14	17 (13.2%)	5	0	0.032 (±0.095)	0.053 (±0.147)				
NDB	22	22 (17.1%)	5	0	0.036 (±0.099)	0.060 (±0.151)				
GIE	12	7 (5.4%)	2	1	0.012 (±0.055)	0.020 (±0.088)				
SIE	15	8 (6.2%)	3	0	0.011 (±0.050)	0.020 (±0.084)				
Total	**83**	**41 (31.8%)**			**0.048 (±0.114)**	**0.081 (±0.171)**				

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

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

	RZA	KAM	NDB	GIE	SIE
RZA		0.706	0.617	0.823	0.128
KAM	0.229		4.903	0.612	0.357
NDB	0.341	0.099		0.584	0.452
GIE	0.124	0.342	0.387		0.193
SIE	0.823	0.607	0.549	0.750	

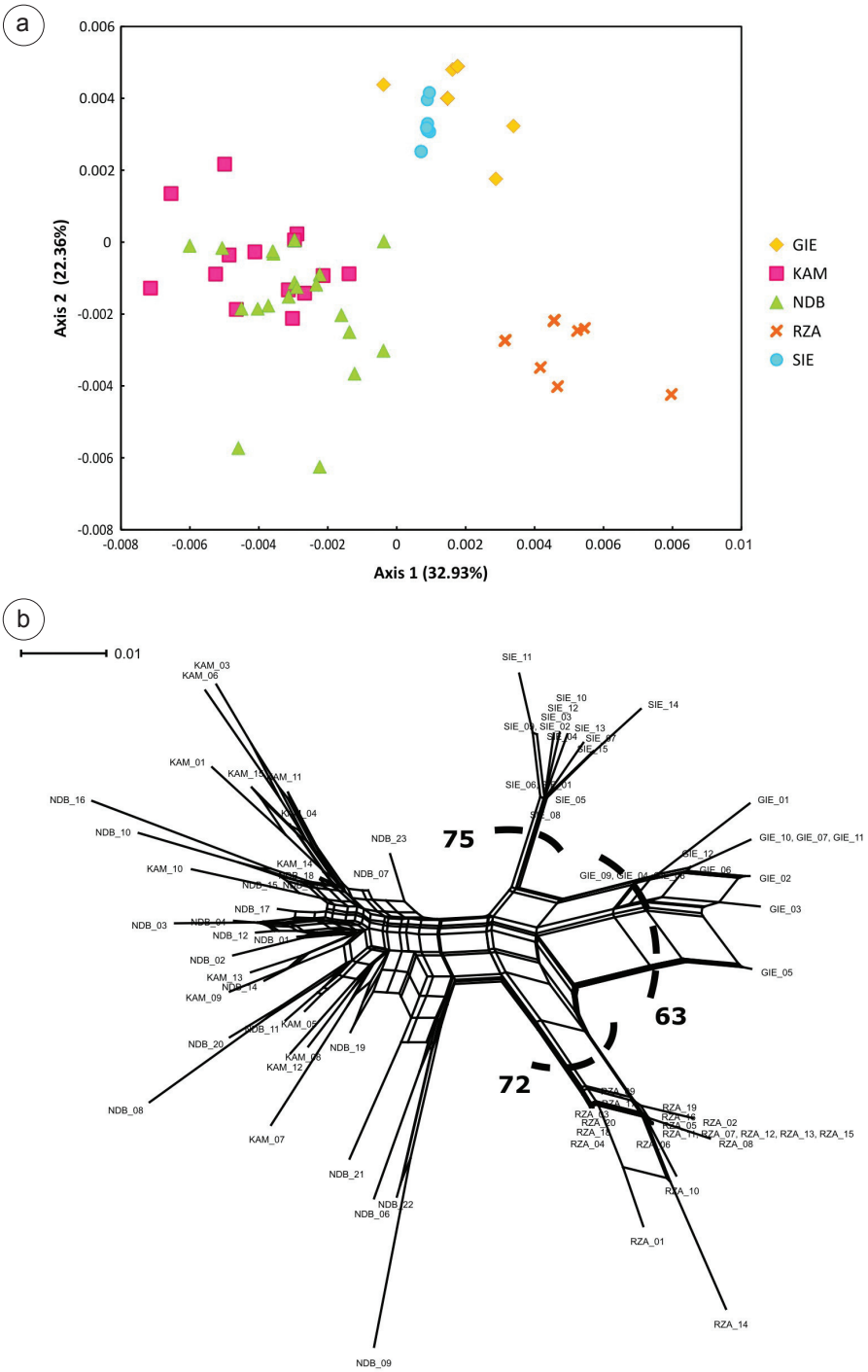

Fig. 2. Intra- and interpopulational genetic diversity of 83 individuals of *Viola uliginosa* from five populations based on AFLP results. For population acronyms see Table 1. (**a**) – Principal Coordinate Analysis (PCoA) (first two axes). Percentage of total variation for each component is given in 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 equalling 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 $P < 0.001$).

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices
		***V. uliginosa*: all populations studied**			
Among populations	4	147.11	2.157	59.99	
Within populations	78	112.19	1.438	40.01	F_{ST} = 0.600*
Total	**82**	**259.30**	**3.595**	**100.00**	
		***V. uliginosa*: three-group division**			
Among groups	2	112.07	1.341	34.95	F_{CT} = 0.350 P = 0.057
Among populations within groups	2	35.05	1.057	27.55	F_{SC} = 0.423*
Within populations	78	112.19	1.438	37.50	F_{ST} = 0.625*
Total	**82**	**259.30**	**3.836**	**100.00**	
		***V. uliginosa*: two-group division**			
Among groups	1	59.21	0.657	17.70	F_{CT} = 0.177 P = 0.102
Among populations within groups	3	87.42	1.749	47.12	F_{SC} = 0.573*
Within populations	78	101.83	1.305	35.18	F_{ST} = 0.648*
Total	**82**	**248.45**	**3.711**	**100.00**	

(1.00 and 0.59, respectively), and much lower in NDB and KAM populations (0.18 and 0.09, respectively) (Table 1).

DISCUSSION

Total gene diversity (H_T) 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. grayi*, 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 Φ_{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. cazorlensis*, *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 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 (Małobęcki 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 Θ 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 cleistogamously-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 → RZA → GIE → KAM vs. GIE → RZA → NDB → 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 *V. 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 geographically 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_j = 0.009), in accordance with the results of Cieślak 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 (Cieślak et al., 2006).

Although the presence of numerous individuals was observed at some locations (eg., 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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