Reproduction and diversity of the endangered *Salix lapponum* L. populations in Eastern Poland

Magdalena POGORZELEC[1],*, Katarzyna GŁĘBOCKA[2], Barbara HAWRYLAK-NOWAK[3], Marzena PARZYMIES[4]

[1]Department of General Ecology, Faculty of Horticulture and Landscape Architecture, University of Life Sciences in Lublin, Lublin, Poland
[2]Institute of Plant Genetics, Breeding and Biotechnology, Faculty of Agrobioengineering, University of Life Sciences in Lublin, Lublin, Poland
[3]Department of Plant Physiology, Faculty of Horticulture and Landscape Architecture, University of Life Sciences in Lublin, Lublin, Poland
[4]Department of Ornamental Plants and Landscape Architecture, Faculty of Horticulture and Landscape Architecture, University of Life Science in Lublin, Lublin, Poland

Abstract: The main aim of the study was to increase the knowledge on the ecology and biology of the boreal relict *Salix lapponum* L., an endangered plant in Poland, in order to create an effective protection program. An important aspect was to determine the within- and among-population genetic diversity of the studied species in order to evaluate whether the basic stages of the generative reproduction cycle in the populations were correct. The results confirmed that *S. lapponum* pollen viability and germinability, for both fresh and stored pollen, were high and so were the seed germination ability and dynamics. All individuals were of sexual origin and the expected heterozygosity was moderate. In spite of this, young individuals were observed in only 1 out of 5 studied populations and it was the most numerous one. Although the genetic diversity among the populations was not very high ($\Phi_{PT} = 0.12$), it accumulated in a quite short period of time, probably during the second part of the 20th century, as a consequence of habitat fragmentation. A lack of gene flow, either by pollen or seeds, may lead to further differentiation of the populations and extinction of the smallest ones. For this reason, preventive measures should be undertaken in order to enlarge the existing populations and to reconstruct *S. lapponum* populations in selected sites where environmental conditions are the most similar to those preferred by this species.

Key words: *Salix lapponum* L., genetic diversity, pollen viability, seed germination, conservation, inter-simple sequence repeat

1. Introduction

More than 15% of Polish flora species are considered rare and endangered. A large group consists of species inhabiting natural peat bogs ecosystems and among them boreal relict species have a significant proportion. On a global scale, these are often plants of a wide geographical range, although in the territory of Poland they exist in isolated small populations on the border of their range (Kaźmierczakowa and Zarzycki, 2001).

The unique susceptibility of wetlands to disturbances caused by anthropopressure and their fragmentation are reflected in the condition of populations of species specific to this type of habitat. A decrease in the number of stands and populations of relict boreal plants in peat bogs is currently a noticeable issue in the context of conservation of the biodiversity of these ecosystems in Poland. The processes leading to diminishing populations of the relict species, and even their complete disappearance from their natural stands, although not clearly exhibited, require an immediate intervention involving the development of programs for their conservation.

Salix lapponum L. (downy willow, family Salicaceae Mirb.) is one of the relict species on the edge of extinction in Poland. This is a medium height shrub growing up to 2 m. The downy willow is a perennial nanophanerophyte plant. It is dioecious and the flowers are borne in female and male catkins that are bicolored, bright purple on the underside and rust-black on the upper side. *S. lapponum* blooms from April to July in the center of its range and from March to May in Eastern Poland. It is an entomophilous and melliferous species. The fruit is a hairy capsule that dehisces into valves. Seeds are dispersed mainly by wind (Piękoś-Mirkowa and Mirek, 2006).

S. lapponum prefers sites that are sunny or partially shaded, wet and moist oligotrophic or mesotrophic habitats with low pH (pH 4–6) and abundant in organic matter—marshy soils in fens, less frequently transitional bogs. It grows individually or in small clusters (Fijałkowski and Lorens, 1998; Zarzycki et al., 2002; Pogorzelec, 2008).

* Correspondence: magdalena.pogorzelec@up.lublin.pl

S. lapponum is a species commonly present in subarctic and boreal peatlands of northern and northeastern Europe as well as western Siberia. Isolated populations are also encountered in some mountain ranges of Central and South Europe and in Scotland (Kaźmierczakowa and Zarzycki, 2001; Podbielkowski, 2002).

In lowland areas in Poland, *S. lapponum* occurs mainly on the eastern side of the Vistula River. Currently, its stands are located in the Łęczna-Włodawa Lakeland (5 stands) in the Polesie National Park, Biebrza National Park, and in Knyszyńska Forest (Pogorzelec, 2009; Pogorzelec, 2010). Two populations grow in the subalpine belt in the Karkonosze Mountains as an endemic association (*Salicetum lapponum* W. Mat. 1965).

As a glacial relict, *S. lapponum* has the status of endangered species (EN) according to the *Polish Red Book of Plants*, or vulnerable (VU) according to the International Union for Conservation of Nature Red List (Kaźmierczakowa and Zarzycki, 2001; Mirek et al., 2006). The species should be subjected to immediate conservation in order to restore adequate levels of the populations' distributions that still exist in Poland.

The present study was undertaken in Eastern Poland (Łęczna-Włodawa Lakeland) aiming to collect as much information as possible about the biology and ecology of *S. lapponum* in order to create an effective program of protection for this species. This study primarily attempted to determine the dominant form of reproduction of the investigated populations and its effectiveness. For this purpose, an effort was made to determine within- and among-population genetic diversity of *S. lapponum* and whether the basic stages of the generative reproduction cycle in the populations were correct.

2. Materials and methods

2.1. The plant material

Each of the 5 populations growing in the peatland ecosystems of the Łęczna-Włodawa Lakeland was studied (Figure 1). Three of them exist in areas protected by the Polesie National Park—peat bogs adjacent to the lakes Długie D (N51°27.103′ E023°10.299′), Moszne M (N51°27.653′ E023°07.067′), and Karaśne K (N51°25.980′ E023°06.576′), while the other ones are located in a peat bog near Lake Bikcze B (N51°22.724′ E023°02.563′) and in a peat bog near Lake Lubowierek L (N51°25.172′ E023°19.096′). In the years 2012–2013, the most numerous was population B (301 individuals), populations M and K consisted of 79 and 31 specimens, respectively, while the smallest ones were populations D (14) and L (9). During the 2 years of observation, flowering and fruiting of *S. lapponum* individuals were only found in population B.

2.2. Pollen viability and germinability

S. lapponum pollen was collected when the plants were in full bloom (April 2012 and 2013, air temperature 10–15 °C). The material was taken from the plants growing in the peat bog near Lake Bikcze (B). One whole inflorescence was collected from each of 10 male individuals (marked with labels) every year. Pollen collected in 2012 was stored in a refrigerator at –18 °C for 1 year.

The germination tests of fresh pollen grains and pollen grains stored for 12 months were performed on microscope slides with 1% agar medium containing 5% glucose solution and an addition of 0.001% boric acid (Diaz and Garay, 2007; Asma, 2008). Pollen grains were placed on glass rods in petri dishes filled with filter paper soaked with distilled water and stored in a refrigerator (11 °C). Then, after 3 h, the slides were analyzed under a light microscope. A total of 1000 pollen grains were counted on
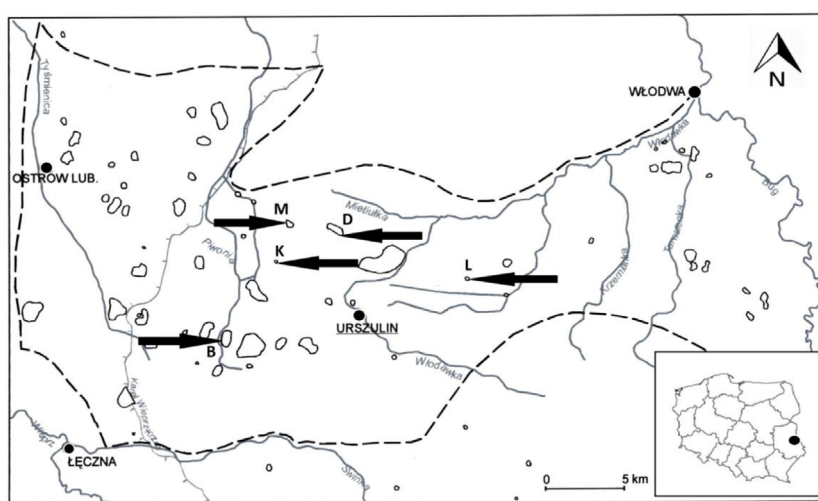

Figure 1. Location of the studied *Salix lapponum* populations in Eastern Poland.

each slide (10 slides with fresh pollen and 10 slides with stored pollen) and the percentage of pollen grains with germinated pollen tubes was determined.

Then smear preparations stained with 2% acetocarmine solution were prepared (Nassar et al., 2000; Lyra et al., 2011) and after 1 h they were analyzed under a light microscope. Fully stained pollen grains filled with cytoplasm were considered viable, whereas unstained pollen grains or pollen grains only partially filled with cytoplasm were considered nonviable. In total 20 × 1000 pollen grains were analyzed. The percentage of viable pollen grains was determined in the examined preparations.

2.3. The seed germination ability and dynamics
The research was conducted on the plant material collected from 10 fruiting specimens of *S. lapponum* in May 2013. The seeds came from plants growing in the peat bog near Lake Bikcze (B).

The germination process was carried out in a laboratory. Petri dishes 10 cm in diameter were filled with 3 layers of filter paper soaked in 2-fold diluted Hoagland II medium (Hoagland and Arnon, 1950). The dishes prepared in this way were sterilized for 10 min in a UV chamber with laminar flow of sterile air. Fifty seeds were placed per dish. The seeds were incubated in a thermostatic chamber under controlled humidity (60%–65%) at a temperature of 20 °C for 10 days. The number of germinated seeds was checked every 24 h. The root and shoot length of seedlings was measured on the 10th day.

2.4. Genetic analyses
Tissue for DNA isolation was collected in 2011. From each plant 2 or 3 leaves were collected and they were kept at

a temperature of –80 °C. The DNA isolation procedure was performed according to Porebski et al. (1997) with modifications and the DNA concentration was assessed on a Nanodrop ND-1000 spectrophotometer.

For DNA amplification, inter-simple sequence repeat (ISSR) primers were used (Zietkiewicz et al., 1994). Six primers, out of 12 initially checked, yielded polymorphic products and were used for analysis (Table 1). Reactions were prepared in 15 µL of solution that contained: 1X PCR buffer (10 mM Tris pH 8.8; 50 mM KCl; 0.08% Nonidet P40) (Thermo Scientific, Lithuania), 200 µM of each dNTP; 500 nM primers; 2.5 mM $MgCl_2$; 60 ng of DNA template; 0.7 U of *Taq* DNA Polymerase (Thermo Scientific, Lithuania). Reactions were developed on a Biometra thermocycler. The following thermal profile was applied: first step of initial denaturation: 95 °C for 7 min; 38 cycles: 95 °C for 30 s with an annealing temperature of 54 °C during the 3 initial cycles, while during the next 3 cycles: 53 °C and the other 32 cycles of 52 °C for 45 s each, primer extension lasted for 2 min in 72 °C; after all the cycles, the final extension lasted 7 min in 72 °C. All amplification products were separated on 2% agarose gels with the addition of 0.01% ethidium bromide.

The presence or absence of an amplification product was treated as a single trait and it was assigned the value of 1 or 0, respectively. In population D, bands were visible in only 4 out of 7 individuals, and so it was decided this group would be removed from the analysis. The final number of individuals and populations is presented in Table 2.

In order to find specimens with very similar or identical genotypes that could be clones, GenoType

Table 1. Sequences of ISSR primers that were used for the analysis of *S. lapponum*. Y = T or C.

Primer name	Sequence	Primer name	Sequence
SR11	$(AC)_8G$	SR28	$(TG)_8G$
SR16	$(GA)_8C$	SR32	$(AG)_8YT$
SR22	$(CA)_8G$	SR68	$(AC)_8T$

Table 2. Within-population genetic diversity estimated by ISSR.

Population	Population size	Number of individuals	Number of bands	% of polymorphic products	Expected heterozygosity (He)
K	39	27	158	98.7	0.175
M	79	27	183	98.9	0.187
B	301	34	157	100.0	0.143
L	9	9	130	97.7	0.182

software (Meirmans and Van Tienderen, 2004) was used. This software compares loci of all pairs of individuals and gathers in separate groups individuals that have the same number of band differences. They are shown in the form of a histogram in the output file.

GeneAlex 6.41 (Peakall and Smouse, 2006) software was used to estimate within- and among-population diversity. The percentage of polymorphic products and expected heterozygosity (H_e) were assessed for each population. To evaluate the proportion of interpopulation variation, analysis of molecular variance (AMOVA) was performed and the Φ_{PT} measure, which is an equivalent of F_{ST} for dominant markers, was evaluated. Based on the binary genetic distance, principal coordinate analysis (PCoA) was conducted in order to depict the diversity among the populations. The correlation between geographic distance and pairwise population differentiation (Φ_{PT}) was assessed by the Mantel test. The significance of AMOVA and Mantel tests was checked by performing 1000 permutations.

STRUCTURE 2.3.4. (Pritchard et al., 2000) software was used to analyze genotypes of all individuals and to connect them into genetic clusters based on the Bayesian approach. This procedure is helpful to check if geographic populations are in agreement with the genetic ones. The software parameters were as follows: 50,000 burn-in, 100,000 iterations of Markov chain Monte Carlo (MCMC), admixture, and correlated allele frequencies models. The number of possible clusters to check (K) was set from 1 to 7 according to the recommendations of Evanno et al. (2005) and 10 replicates were done for each K. The STRUCTURE output files did not show clearly the most possible number of clusters and therefore a procedure based on the second-order rate of change of the likelihood function (ΔK), described by Evanno et al. (2005), was adopted. This procedure was run by the on-line program Structure Harvester (Earl and vonHoldt, 2012).

3. Results
3.1. Pollen viability and germinability
The viability of fresh pollen grains ranged from 95% to 99.3% (97.2 ± 13.57), whereas this parameter was slightly reduced in the case of pollen stored for 12 months, ranging between 88.6% and 98.8% (94.65 ± 2.93). The germinability was also higher in the case of fresh pollen (17.5% ± 10.55 on average) in comparison to stored pollen grains (10.8% ± 5.81).

3.2. The seed germination ability and dynamics
Properly germinated seeds were observed in the first 24 h of the experiment (49% of the total number of seeds sown). The highest increase in this parameter was observed between the 1st and the 2nd day of the experiment. On the 4th day of the experiment, the number of germinating seeds stabilized and no further increase in the number of seeds germinating per day was observed. The germinability of seeds, estimated on the basis of the results obtained after 10 days of the experiment, was 71%. The mean seedling shoot length on the 10th day of the experiment was 0.822 cm (±0.182), while the mean root length was 0.188 cm (±0.098).

3.3. Genetic analyses
One hundred and ninety amplification products were obtained by ISSR analysis (on average 31.67 products per primer), but some of them were absent in some populations. Population L had the least number of bands, while population M had the highest one (Table 2) with 2 private ones. When taking into account all the populations, all amplification products were polymorphic, but, considering them separately, there were 2 or 3 monomorphic bands in each, except population B. The expected heterozygosity ranged from 0.143 (population B) to 0.187 (population M).

The histogram created by GenoType software was unimodal (Figure 2). The least number of loci differences was 16 and the highest one was 88 (2 pairwise comparisons
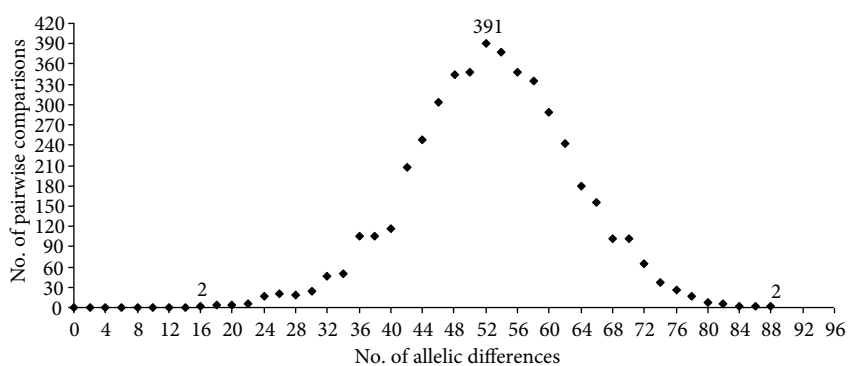

Figure 2. A histogram of the number of allele differences for pairwise comparisons for *S. lapponum* individuals based on GenoType results.

in each case). A peak on the histogram was observed for 51 and 52 band differences and was attributed to 391 pairs of individuals. When identifying clones by this method, an assumption is made that clones can differ by 1 or 2 products, depending on the adopted threshold. Individuals that differ by more products are regarded as distinct genotypes of sexual origin. The reason for genotypic heterogeneity of clones could be related to somatic mutations, sample contamination by microorganisms or fungi, or errors during amplification (Douhovnikoff and Dodd, 2003). As in the histogram no peak was observed for 1 or 2 loci differences, it was stated that there were no clonal individuals and no analysis was made for any of the populations separately.

Based on AMOVA software, among-population diversity was 12% (Φ_{PT} = 0.12) (Table 3). Most of the variation (88%) occurred within populations and for this reason PCoA was prepared according to the binary genetic distance of individual plants (Figure 3). In this plot, specimens of population K were grouped together and separated from the other 3 populations. Populations M and B assembled as 2 overlapping clusters with individuals of population L interspersed.

On the basis of the Mantel test, interpopulation genetic diversity and geographic distance were barely correlated (r = –0.091) and the results were statistically nonsignificant after 1000 permutations performed (P = 0.380).

The results obtained from STRUCTURE were analyzed by the Structure Harvester and it turned out that the maximum value of ΔK was for K = 3 (Figure 4). Populations

M and L had a similar genetic background and for this reason they were considered as one cluster. Moreover, these 2 populations were also genetically similar when K = 4 (data not shown). Although the clusters M+L and B were diverse, they all had individuals with mixed ancestry. Parallel to the PCoA plot, population K was genetically homogeneous, clearly distinct from the others, and the contribution of clusters M+L and B was restricted.

4. Discussion

The family Salicaceae, with the genera *Salix* and *Populus*, includes species that are able to propagate generatively and vegetatively. The method of propagation depends on the species and environmental conditions. In a specific habitat, new specimens may develop from seeds or somatic tissue fragments, depending on the season and soil conditions. In the case of endangered species, it is very important to establish conditions suitable for their sexual propagation (Saunders and Sipes, 2006). The effectiveness of generative propagation of species belonging to the genus *Salix* depends on a few factors related to individual and population features. Because of the fact that they are diclinous, a significant role is played by the sexual structure of the population, which is usually feminized at a level of 4:1. Not always can the ratio between female and male individuals be observed due to irregular flowering and at present there are no molecular techniques that would allow us to distinguish plants of different sexes (Zasada et al., 2008).

Table 3. Partitioning of genetic variation based on binary genetic distance using AMOVA. P < 0.001.

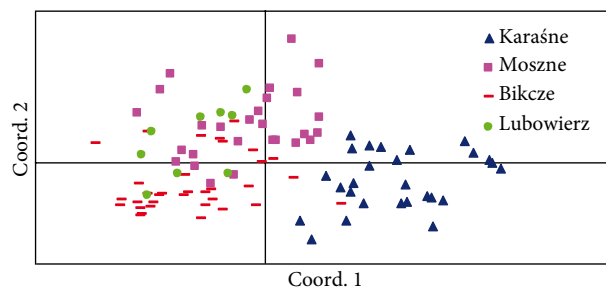| Source	d.f.	Sum of squares	Mean sum of squares	Variance	Percentage of variation
Among populations	3	301.868	100.623	3.333	12%
Within populations	93	2206.854	23.730	23.730	88%
Total	96	2508.722		27.062	100%

Figure 3. Principal coordinate analysis (PCoA) of 4 populations of *Salix lapponum* from Poland.
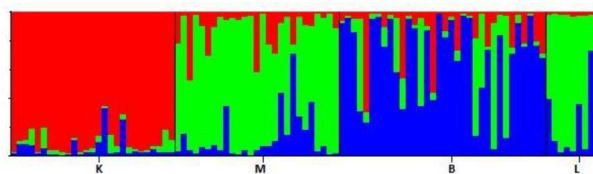

Figure 4. The structure assignment of 4 Polish *Salix lapponum* populations for K = 3. Each color represents a genetically diverse background and black vertical lines separate the populations.

Two main phases in the reproductive cycle of plants are noteworthy and they might be used in planning species conservation. Any disturbance of them may cause a decrease in propagation effectiveness. Pollen viability and germinability may be used as an indicator of correctness of sexual processes and adaptation abilities of plants, which is used in planning active species protection (Tangmitcharoen and Owens, 1997; Dane et al., 2004; Diaz and Garay, 2007; Khan and Perveen, 2008; Skalona et al., 2010; Lyra et al., 2011). The results of the experiments conducted in 2012 and 2013 indicate the high viability and germinability of *S. lapponum* pollen obtained from population B specimens. In vitro culturing of *S. lapponum* pollen may soon become one of the key stages of active protection of this species, thanks to which it will be possible to preserve the gene resources of the small populations that still exist in Eastern Poland.

The second key step in the reproductive cycle of plants is production of seeds and their germination ability and dynamics are the most important characteristics. Douhovnikoff et al. (2005) analyzed populations of *Salix exigua* L. along 2 rivers: the first one was regulated with low water disturbance, while the second one was unregulated, which resulted in floods on the river banks. Seedlings were more often detected in the disturbed area. Smulders et al. (2008) and Sochor et al. (2013) observed similar results when studying populations of *Populus nigra* L. and *S. daphnoides* Vill., which were located on regulated and unregulated riversides. According to Douhovnikoff et al. (2005) and Sochor et al. (2013), seeds of the above-mentioned willow species are characterized by low viability and they germinate in soil that is both moist and rich in nutrients. Thus, flooded areas are suitable for seedling germination that can reach even 100%, but, on the other hand, a large amount of water followed by periods of drought causes high seedling mortality in the first year. In spite of all these obstacles, some seedlings survive in years with more favorable conditions and they grow into mature plants that, because of their sexual origin, enhance the genetic diversity of a population. Mosner et al. (2012) studied another willow species, *S. viminalis* L., and their results were contrary: the water flow caused the plants to bend, which promoted clonal recruitment of the submerged parts.

Based on the genetic analysis, there were no clones in the *S. lapponum* populations in the Łęczna-Włodawa Lakeland. It was also observed that seeds collected from population B were of high germination ability and dynamics in the laboratory conditions. However, there were no seedlings observed in the natural habitat, which was probably caused by environmental factors. Stamati et al. (2007) investigated

3 willow species and *S. lapponum* was one of them. These authors also did not find any seedlings of this species, although individuals had multilocus genotypes, which is unusual for clonally propagated plants. Among 224 *S. lapponum* individuals, there were only 13 cases of plants of identical or very similar genotypes. Although species of the genus *Salix* tend to disperse by vegetative propagules, these findings indicate that this specific willow species reproduces mainly sexually, despite its unstable habitat. Stamati et al. (2007) also gave 2 possible explanations for poor seedling establishment. Firstly, because of the high mortality, seedlings occur incidentally and they can easily pass unnoticed during observations, while the second explanation assumes that a large quantity of seeds is produced once in a few years.

The results obtained during the laboratory experiments conducted in the period 2012–2013 do not exclude the possibility to cultivate *S. lapponum* plants germinated in vitro. Population B, as the most numerous one in the study area, may become a source of plant material to establish ex situ protection of *S. lapponum*.

The percentage of polymorphic products in all the populations analyzed was rather equal and far higher than in other studies on peripheral or isolated populations of other plant species (Smulders et al., 2008; Grossi et al., 2011; Sochor et al., 2013). Although the expected heterozygosity (H_e) ranged from 0.143 (population B) to 0.187 (population M) and these values were quite similar to the results obtained in other studies using dominant markers (Smulders, 2008; Sochor et al., 2013), they were still lower in comparison to codominant markers (Kikuchi et al., 2011; Lee et al., 2011; Sochor et al., 2013). Nybom (2004) stated that H_e of endemic and peripheral populations should be greater than 0.20, but at the same time she pointed out that heterozygosity is lower for species whose seeds disperse and germinate close to maternal plants, which was observed in the studied populations of *S. lapponum*. Pospíšková and Sálková (2006) noted that genetic diversity was lower for young plants of *Populus nigra* and it increased with the age of individuals. In our study, the only population where young specimens were observed was population B and the expected heterozygosity was clearly lower for this group when compared with the other 3 populations, although individuals were not divided into age cohorts. However, according to the standard established by Nybom (2004), genetic diversity of populations K, M, and L was rather low. It should be also emphasized that genetic diversity and overall conditions of populations remarkably depend on the number of individuals. In small populations, closely related plants mate, which leads to increased homozygosity

and inbreeding depression (Bossuyt, 2007). Furthermore, selection pressure is weaker in such populations than in large ones and therefore deleterious recessive alleles are fixed (Reed, 2005). The accumulation of such factors prevents populations from increasing their quantity and they are doomed to extinction. Population B was the largest of all the studied ones (301 individuals) and the only one with young plants; hence it could be assumed that it was in good condition. The other 3 populations were much smaller and, which was the most disturbing, they consisted only of mature plants.

The diversity was not extremely high among the populations (Φ_{PT} = 0.12, P = 0.001). This may lead to the conclusion that gene flow between the populations was not very intense. In the PCoA plot, populations B, M, and L overlapped and STRUCTURE showed that the most probable number of populations was 3, because M and L were combined into 1 cluster.

It is generally assumed that genetic diversity among willow populations is rather low. Seeds are small and light, so that they can be easily carried for long distances by wind (Hörandl et al., 2002). However, Kikuchi et al. (2011) obtained contrary results when studying *Salix hukaoana* Kimura populations, where gene flow was quite intense only in a group of populations located in the upper reaches of the river Yubiso. Similarly, Sochor et al. (2013) recorded rather high and significant diversity among *S. daphnoides* Vill. populations. Furthermore, they discovered that 1 population consisted in fact of 2 groups of individuals that were 130-m apart but they were highly isolated and poor gene flow led to considerable diversity. It should also be mentioned that genetic variation is created not only by gene flow but also by other factors, such as the ability to colonize new territories and to remain there, habitat fragmentation, or environmental heterogeneity (Wróblewska, 2013). In the case of *S. lapponum*, gene flow is unlikely either at the pollen level (this species is insect-pollinated and distances between populations are from 2 to almost 12 km) or by seeds (they are prone to be carried by wind for long distances, but because of the specific phytocoenosis structure they usually fall close to maternal plants).

According to the historical data, until the 50s of the 20th century the research area (Łęczna-Włodawa Lakeland) was uniform and marshy and *S. lapponum* was present in large quantities (Fijałkowski, 1958). The populations that remain there are isolated due to the fragmentation of their natural habitats resulting from the changes caused by hydraulic improvements carried out for many years in

this area (Michalczyk, 1994). In the light of these data, the homogeneity of the genetic structure of populations M and L requires a new approach. It is probably a result of the much more vast range of the *S. lapponum* population in this area in the past than an outcome of gene flow through seeds or pollen. The lack of correlation between the distance and genetic diversity also confirms the isolation of the population. The absence of gene flow may lead to greater and greater differentiation of the population; this, in combination with the reduced ability of *S. lapponum* to propagate within the area of Eastern Poland, makes the species endangered and requires immediate preventive measures.

4.1. Implications for conservation

The moderate intrapopulation diversity of *S. lapponum* and the lack of disturbances at the basic stages of the propagation cycle might be of key importance for an effective implementation of protection measures in order to preserve this species in the territory of Eastern Poland. At the moment, the biggest problem is the small number of the remaining isolated populations as well as their differentiation and usually their small quantity.

The foundation for preventive measures should be the enlargement of existing populations and reconstruction of *S. lapponum* populations in selected sites where environmental conditions are the most similar to those preferred by this species. Such activities should be undertaken as quickly as possible since there is a constant decrease in numbers and there are no new individuals in the populations. Conservation should be in the first step based on ex situ cultivation of the species. Individuals intended for reintroduction or strengthening the population should be grown in laboratory conditions from seeds collected from natural habitats. The second step, as an alternative solution, might be in vitro cultivation or vegetative propagation through soft and woody cuttings collected from plants growing in natural habitats. The methods mentioned above are invasive and should be considered only when generative propagation from seeds fails.

References

Asma BM (2008). Determination of pollen viability, germination ratios and morphology of eight apricot genotypes. Afr J Biotechnol 7: 4269–4273.

Bossuyt B (2007). Genetic rescue in an isolated metapopulation of a naturally fragmented plant species, *Parnassia palustris*. Conserv Biol 21: 832–841.

Dane F, Olgun G, Dalgic O (2004). *In vitro* pollen germination of some plant species in basic culture medium. J Cell Mol Biol 3: 71–76.

Diaz L, Garay BR (2007). Simple methods for in vitro pollen germination and pollen preservation of selected species of the genus *Agave*. E-Gnosis 6: 1–7.

Douhovnikoff V, Dodd RS (2003). Intra-clonal variation and a similarity threshold or identification of clones: application to *Salix exigua* using AFLP molecular markers. Theor Appl Genet 106: 1307–1315.

Douhovnikoff V, McBride JR, Dodd RS (2005). *Salix exigua* clonal growth and population dynamics in relation to disturbance regime variation. Ecology 86: 446–452.

Earl DA, vonHoldt BM (2012). Structure Harvester: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4: 359–361.

Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611–2620.

Fijałkowski D (1958). Badania nad rozmieszczeniem i ekologią wierzby lapońskiej (*Salix lapponum*) na Pojezierzu Łęczyńsko-Włodawskim. Fragm Flor et Geobot 3: 89–103 (article in Polish with an abstract in English).

Fijałkowski D, Lorens B (1998). Rośliny borealne we florze Lubelszczyzny. Ann UMCS sec. C 53: 61–71 (article in Polish with an abstract in English).

Grossi MA, Julio N, Gardenal CN, Di Rienzo J, Funes G (2011). Genetic variability in *Apurimacia dolichocarpa* (*Fabaceae*), a narrow endemic species of Córdoba Hills, Argentina. Ann Bot Fenn 48: 21–28.

Hoagland DR, Arnon DI (1950). The water-culture method for growing plants without soil. Calif Agric Exp Sta Circ 347: 1–32.

Hörandl E, Florineth F, Hadacek F (2002). Weiden in Österreich und angrenzenden Gebieten. Wien, Austria: Universität für Bodenkultur (in German).

Kaźmierczakowa R, Zarzycki K (2001). Polska Czerwona Księga Roślin. Polish Red Book of Plants. Pheridiophytes and Flowering Plants. 2nd ed. Cracow, Poland: Botany Institute PAN.

Khan SA, Perveen A (2008). Germination capacity of stored pollen of *Morus alba* (Moraceae) and their maintenance. Pak J Bot 40: 1823–1826.

Kikuchi S, Suzuki W, Sashimura N (2011). Gene flow in an endangered willow *Salix hukaoana* (*Salicaceae*) in natural and fragmented riparian landscapes. Conserv Genet 12: 79–89.

Lee KM, Kim YY, Hyun JO (2011). Genetic variation in populations of *Populus davidiana* Dode based on microsatellite marker analysis. Genes Genomics 33: 163–171.

Lyra DH, Sampaio LS, Pereira DA, Silva AP, Amaral CLF (2011). Pollen viability and germination in *Jatropha ribifolia* and *Jatropha mollissima* (*Euphorbiaceae*): Species with potential for biofuel production. Afr J Biotechnol 10: 368–374.

Meirmans PG, Van Tienderen PH (2004). GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol Ecol Notes 4: 792–794.

Michalczyk Z (1994). Zmiany sieci hydrograficznej w rejonie oddziaływania kanału Wieprz-Krzna. In: Radwan S, editor. Środowisko przyrodnicze w strefie oddziaływania kanału Wieprz-Krzna. Lublin, Poland: Towarzystwo Wolnej Wszechnicy Polskiej, pp. 43–46 (in Polish).

Mirek Z, Zarzycki K, Wojewoda W, Szeląg Z (2006). Czerwona lista roślin i grzybów Polski. Cracow, Poland: Instytut Botaniki PAN im. W. Szafera (in Polish).

Mosner E, Liepelt S, Ziegenhagen B, Leyer I (2012). Floodplain willows in fragmented river landscapes: Understanding spatio-temporal genetic patterns as a basis for restoration plantings. Biol Conserv 153: 211–218.

Nassar NMA, Santos ED, Sra D (2000). The transference of apomixes genes from *Manihot neusaria* Nassar to cassava, *M. eculenta* Crantz. Hereditas 132: 167–170.

Nybom H (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol Ecol 13: 1143–1155.

Peakall R, Smouse PE (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6: 288–295.

Piękoś-Mirkowa H, Mirek Z (2006). Rośliny chronione. Warsaw, Poland: Multico Oficyna Wydawnicza (in Polish).

Podbielkowski Z (2002). Geografia roślin. Vol I. Europa, Azja, Afryka. Warsaw, Poland: Wydawnictwo Naukowe PWN (in Polish).

Pogorzelec M (2008). Influence of chosen environmental abiotic factors on *Salix lapponum* L. populations functioning in Polesie Lubelskie Region. Pol J Environ Stud 17: 139–144.

Pogorzelec M (2009). The downy willow (*Salix lapponum* L.) as a component of different phytocoenosis in Poleski National Park. Acta Agrobot 62: 107–116.

Pogorzelec M (2010). *Salix lapponum* L. (Downy willow) in stands under anthropopressure in the Łęczna-Włodawa Lakeland. Acta Agrobot 63: 47–53.

Porebski S, Bailey LG, Baum BR (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep 15: 8–15.

Pospíšková M, Sálková I (2006). Population structure and parentage analysis of black poplar along the Morava river. Can J Forest Res 36: 1067–1076.

Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Reed DH (2005). Relationship between population size and fitness. Conserv Biol 19: 563–568.

Saunders NE, Sipes SD (2006). Reproductive biology and pollination ecology of the rare Yellowstone Park endemic *Abronia ammophila* (Nyctaginaceae). Plant Species Biol 21: 75–84.

Skalona D, Vavratilova B, Ondrej V, Lebeda A (2010). Optimizing culture for in vitro pollination and fertilization in *Cucumis dativus* and *C. melo*. Acta Biol Cracov Bot 52: 111–115.

Smulders MJM, Cottrell JE, Lefevre F, van der Schoot J, Arens P, Vosman B, Tabbener HE, Grassi F, Fossati T, Castiglione S et al. (2008). Structure of the genetic diversity in black poplar (*Populus nigra* L.) populations across European river systems: consequences for conservation and restoration. Forest Ecol Manag 255: 1388–1399.

Sochor M, Vašut RJ, Bártová E, Majeskỳ L, Mráček J (2013). Can gene flow among populations counteract the habitat loss of extremely fragile biotopes? An example from the population genetic structure in *Salix daphnoides*. Tree Genet Genomes 9: 1193–1205.

Stamati K, Hollingsworth PM, Russel J (2007). Patterns of clonal diversity in three species of sub-arctic willow (*Salix lanata*, *Salix lapponum* and *Salix herbacea*). Plant Syst Evol 269: 75–88.

Tangmitcharoen S, Owens JN (1997). Pollen viability and pollen-tube growth following controlled pollination and their relation to low fruit production in teak (*Tectona grasndis* Linn. f.). Ann Bot 80: 401–410.

Wróblewska A (2013). High genetic diversity within island-like peripheral populations of *Pedicularis sceptrum-carolinum*, a species with a northern geographic distribution. Ann Bot Fenn 50: 289–299.

Zarzycki K, Trzcińska-Tacik H, Różański W, Szeląg Z, Wołek J, Korzeniak U (2002). Ecological Indicator Values of Vascular Plants of Poland. Cracow, Poland: Instytut Botaniki PAN.

Zasada JC, Douglas DA, Buechler W (2008). *Salix*. In: Bonner FT, Karrfalt RP, editors. The Woody Plant Seed Manual. USA: United States Department of Agriculture, Forest Service.

Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176–183.