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Population History, Genetic Variation and Conservation Status of the Endangered Birch Species *Betula nana* L. in Poland

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The effective conservation of species requires data on the levels and distribution of genetic diversity within and among populations. In this study, we estimated the genetic variation in three isolated populations of *Betula nana* in Poland. An analysis of 11 nuclear microsatellites revealed moderate mean heterozygosities (H_O=0.556, H_E=0.562), low mean number of alleles per locus (A=4.57) and no inbreeding in the total sample. An M-ratio test indicated that each population had experienced a severe bottleneck in the past. Tests for heterozygosity excess revealed that a significant decrease in the numbers of individuals in two populations had occurred quite recently. The large number of private alleles and very restricted number of migrants between populations (Nm=0.35) strongly suggest that genetic drift and geographic isolation are the primary factors responsible for the reduction of genetic variation in the Polish populations of *B. nana*. We detected two cpDNA haplotypes in the study populations, which can be explained in terms of either the genetic drift acting on the relict localities or a postglacial recolonisation from distinct refugia. Palynological data indicated that one refugium could be located in the Carpathians and their northern foreland. The primary threat to *B. nana* in Poland is the overgrowth of its habitats by competing species, which has likely resulted in a lack of generative reproduction in the mountain populations.

Keywords *Betula nana*, conservation genetics, cpDNA, genetic diversity, isolation, microsatellites
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1 Introduction

The number of threatened species increases every year (Vié et al. 2008). To halt this trend, which is dangerous for global biodiversity, the International Union for Conservation of Nature (IUCN) recommends actions to reduce the loss of diversity within species as well as maintain a variety of ecosystems. The effective conservation of species requires data on the levels and distribution of genetic variation within and among populations. When most of the genetic diversity of a species is found within populations, fewer populations must be preserved to represent the full range of variation within the species (Neel and Ellstrand 2003). Conversely, if genetic variation is held primarily between populations, the preservation of a larger number of present-day populations is required to maintain the variation within the species (Neel and Ellstrand 2003). The frequencies of alleles at genetic marker loci could also indicate which populations are priorities for protection. Holsinger and Gottlieb (1991) suggested that, to maintain long-term population viability, a representative sample of moderate- to high-frequency alleles should be preserved. On the other hand, some researchers propose to conserve populations that represent unique alleles, which are usually infrequent, to strengthen the genetic diversity of the species (Gapare et al. 2005, Jadwiszczak et al. 2011a). The latter is true for the rare endemic shrub *Guaiacum unijugum* inhabiting the Cape Region of Baja California. In this species, an analysis of microsatellite polymorphism revealed that populations were almost monomorphic and that weak genetic differentiation was maintained solely by the presence of rare private alleles (McCauley et al. 2010).

A suitable level of genetic diversity within and between natural populations is crucial to cope with environmental changes, thereby determining the probability of long-term survival of the species. Therefore, it is very urgent to evaluate the genetic variation in endangered taxa. Many investigations have demonstrated that rare and threatened plant species might be genetically depauperate compared to widely distributed congeners (Hamrick and Godt 1989, Ellstrand and Elam 1993, Nybom 2004). Genetic erosion in endangered plants may be driven by small population sizes, resulting in increases in random genetic drift, inbreeding and a reduction in gene flow (Ellstrand and Elam 1993). However, a comparative analysis conducted for six endemic shrubs in the south-eastern United States indicated a rather weak effect of population size on some parameters of genetic diversity (Menges et al. 2010). Within-population genetic variation was also not correlated with population size in the endangered perennial herb *Parnassia palustris* in northern France (Bonnin et al. 2002) or in the Alpine plant *Campanula thyrsoides* (Ægisdóttir et al. 2009). The above studies strongly suggest that there is no simple relationship between the level of genetic variation and population size; hence, genetic resources must be estimated directly from the sampled populations.

The dwarf birch, *Betula nana* L. is one of the endangered species in central and eastern Europe (EN category of the IUCN). This cold-tolerant species is widespread in the Arctic regions of Eurasia, Greenland, Iceland and North America. In central and eastern Europe, the dwarf birch is a glacial relict that is still present in several localities, primarily in the Alps and the Carpathians (Kruszelnicki and Fabiszewski 2001). In Poland, *B. nana* occurs in three isolated reserves: "Torfowisko pod Zieleńcem" (Sudety Mts.), "Torfowiska Doliny Izery" (Sudety Mts.) and "Linje" in northern Poland. *B. nana* is a highly branched shrub, usually growing up to 1 m in height. It is a characteristic species of open raised-bog and tundra environments. The dwarf birch, like its congeners, is a monoecious, wind-pollinated and wind-dispersed species. In well-established populations, dwarf birches can reproduce by vegetative layering. It has been suggested that vegetative layering was the primary mode of reproduction in the Polish populations of *B. nana* (Kruszelnicki and Fabiszewski 2001, Ejankowski 2010).

Until now, estimations of genetic diversity in *B. nana* populations have been conducted mostly within the northern part of the species range where populations are not vulnerable. Analyses of the variation in chloroplast DNA (cpDNA) have suggested that the most variable populations are those from northern Norway and the eastern part of Iceland (Palmé et al. 2004, Maliouchenko et al. 2007, Thórsson et al. 2010). The distribution of chloroplast haplotypes suggests that the dwarf birch colonised the Scandinavian Peninsula

from south-eastern and south-western directions (Palmé et al. 2004, Maliouchenko et al. 2007). Iceland was populated from northern Scandinavia in the early Holocene (Thórsson et al. 2010). Except for the "Linje" reserve, where a high level of RAPD marker variation was found (Dąbrowska et al. 2006), there is no data on the genetic diversity in remnant Polish populations of the dwarf birch. The present study has three objectives: 1) to evaluate genetic variability within and between isolated populations of the species using nuclear microsatellites; 2) to estimate the haplotype diversity of cpDNA to describe the phylogenetic origin of Polish populations of *B. nana* and 3) to discuss the genetic results in the context of the current knowledge of the Pleistocene and Holocene history of the dwarf birch in the area of Poland.

Fig. 1. Location of historic and present-day populations of *B. nana* in Poland. Black squares – the Weichselian Pleniglacial sites (1 – Dobra, 2 – Nowa Huta, 3 – Brzeźnica, 4 – Smerek), open squares – the Oldest Dryas sites (5 – Gościąż Lake, 6 – Lednica Lake), black circles – the Older Dryas sites (7 – Pomieczyno, 8 – Niechorze, 9 – Durne Bagno, 10 – the Carpathians), open circles – the Younger Dryas sites (LIN, 11 – Wieluń, 12 – the Knyszyńska Forest), grey circles – the Preboreal sites (13 – Miłkowskie Lake, 14 – Długie Bagno), asterisks – the Boreal sites (IZER, ZIEL). Still existing populations are underlined.

2 Material and Methods

2.1 Study Populations

B. nana was sampled from three Polish reserves: "Linje" (site code LIN) in the Pomorze region (northern Poland), "Torfowiska Doliny Izery" (site code IZER) and "Torfowisko pod Zieleńcem" (site code ZIEL) in the Sudety Mts. (south-western Poland). The population names and their locations are given in Table 1 and Fig. 1. The two mountain localities are approximately 85 km apart from one another, and both are located approximately 330 km from the lowland population in northern Poland. The nearest populations outside the Polish borders are in Germany and the Czech Republic (Kruszelnicki and Fabiszewski 2001). The Polish populations of the dwarf birch are of different sizes. The largest is the LIN population because it consists of a few thousand individuals (Kruszelnicki and Fabiszewski 2001). The mountain populations are clearly smaller, though a few thousand individuals in IZER were also suggested to exist (Matuła et al. 2000). Our field observations, made in 2011, revealed that the mountain populations could comprise several dozen individuals at most. At the LIN site, *B. nana*

Table 1. Location and sampling information for three Polish localities of *B. nana.*

Population name	Population code	Geographical position		No. samples collected	No. genets analysed	cpDNA haplotype [a)]
		Latitude	Longitude			
Linje	LIN	53°11´N	18°18´E	20	17	I
Torfowiska Doliny Izery	IZER	50°51´N	15°21´E	20	18	II
Torfowisko pod Zieleńcem	ZIEL	50°20´N	16°25´E	20	17	I

[a)] Description of haplotypes in Table 2.

grows in the central part of the raised bog, where it has to compete for light with *Ledum palustre* and *Vaccinium uliginosum*. In the mountain populations, a dominant type of the vegetation community is *Pino mugo-Sphagnetum*; however, the dwarf birch occurs in some patches not covered by *Pinus mugo*. *Molinia caerulea* is another competitive species that displaces small individuals of *B. nana* in drier parts of the habitat at IZER (Matuła et al. 2000).

2.2 Sampling and DNA Analyses

We randomly sampled 20 branches from each of the three populations. To minimise the chance of collecting vegetative ramets, sampled individuals were separated by at least 20 m in the LIN population and at least 1 m at the much smaller Sudety Mts. localities. Only fresh and healthy leaves were collected. Leaves were immediately placed in silica gel granules for field storage and transport to the laboratory. The collection of material was approved by the Regional Directors of Environmental Protection in Bydgoszcz (WPN.6205.43.2011.KLD) and Wrocław (WPN.6205.59.2011.MR).

Leaf material was manually crushed in liquid nitrogen. Total DNA was extracted using an AX Plant Kit (A&A Biotechnology) according to the manufacturer's protocol. Samples were screened using 11 nuclear microsatellites originally designed for *B. pendula* (L1.10, L2.7, L13.1, L5.4, L5.1, L3.1, L2.3, L022; Kulju et al. 2004) and *B. pubescens* ssp. *tortuosa* (Bo. G182, Bo.F394, L021; Truong et al. 2005). The arrangement of primers in four PCR multiplexes, the PCR profile for each multiplex and the content of PCR reactions were according to Jadwiszczak et al. (2011a). Fluorescently labelled PCR products were analysed on the ABI PRISM 3130 sequencer (Applied Biosystems) and scored using GeneMapper 4.0 (Applied Biosystems) analysis software. Each of the distinct multilocus genotypes was assumed to be a distinct genet. However, we revealed two pairs of samples in each population with the same genotype. This indicates that vegetative ramets were collected. Moreover, we also found one individual at both the LIN and ZIEL reserves having three alleles at some loci. Vegetative ramets and specimens with three alleles were excluded from the statistical analyses. In total, 52 individuals were studied (Table 1).

After genotyping the nuclear loci, three non-coding cpDNA fragments (AS, CD and TF) were amplified for each genetic individual with primer pairs designed by Taberlet et al. (1991) and Demesure et al. (1995). The analysis of cpDNA variation was conducted using the PCR-RFLP method. PCR reactions and restriction procedures followed Jadwiszczak et al. (2012). PCR-RFLP fragments were separated by electrophoresis on horizontal 1.5% agarose gels in 1xTBE buffer and stained with ethidium bromide. An O'Range Ruler 50bp DNA Ladder (Fermentas) was run on each gel as a size marker. Banding patterns were observed under a UV-camera (BioRad) and analysed by careful manual verification at least twice.

2.3 Statistical Methods

MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) was used to find potential genotyping errors, i.e., microsatellite null alleles, large allele dropouts and stuttering. The proportion of polymorphic loci (P), the mean number of alleles per locus (A) and the mean effective number of alleles per locus (A_E) calculated as $A_E = 1/(1 - H_E)$ for each locus and averaged across loci, as well as the average observed (H_O) and expected (H_E) heterozygosities, were calculated using ARLEQUIN 3.5 (Schneider and Lischer 2009) and FSTAT 2.9.3 (Goudet 1995). The frequencies of microsatellite alleles were estimated using GENEPOP 4.1 (Raymond and Rousset 1995), and then the number of private alleles (N_{PA}) in every locus and population was counted. Departures from Hardy–Weinberg equilibrium (HWE) in each population were tested using an exact test of HWE with a Markov chain algorithm with GENEPOP. Tests for linkage disequilibrium (LD) for all pairs of loci within each population and over all populations were carried out with the help of GENEPOP, which generates exact probabilities of a type-I error for the null hypothesis that a pair of loci is unlinked. The level of inbreeding in each population was measured using the inbreeding coefficient (F_{IS}). The statistical significance of F_{IS} was established in FSTAT using 5 000 repli-

cates generating 95% confidence intervals (CI). A sequential Bonferroni procedure was applied to multiple comparisons to keep the experimentwise error rate to a specified level (Rice 1989). The theoretical numbers of migrants entering every population per generation (Nm) were estimated by the private allele method using GENEPOP.

We used two methods to detect potential reductions in population sizes. The program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996, Piry et al. 1999) was used to test for a recent reduction of effective population size under the infinite allele model (IAM), stepwise mutation model (SMM) and two-phased mutational model (TPM). In the TPM model, 70% single-step mutations were chosen. The significance of heterozygosity excess under IAM, SMM and TPM was estimated using the one-tailed Wilcoxon test (Cornuet and Luikart 1996). Recently bottlenecked populations show an excess of heterozygosity relative to that expected based on the number of alleles. The program M_P_Val was used to estimate M value, which is the mean ratio of the number of alleles to the total range in allele size (Garza and Williamson 2001). The M-ratio is smaller in a bottlenecked population than in an equilibrium population. Moreover, the M-ratio test is more powerful at detecting older and more severe declines than the method implemented in BOTTLENECK (Williamson-Natesan 2005).

Measures of multilocus differentiation among populations (F_{ST}) were calculated in FreeNA using the ENA method (Chapuis and Estoup 2007) for nuclear microsatellites, whereas ARLEQUIN was used for cpDNA. The ENA method corrects the values of F_{ST} under the presence of null alleles. F_{ST} calculations were conducted only for loci that showed a lack of LD in the entire data set. For the F_{ST} values, 95% CIs were estimated with 10 000 bootstrap replicates over all loci. The genetic similarity of the studied populations was also tested using a Bayesian clustering method implemented in STRUCTURE 2.3.3 software (Pritchard et al. 2000). For each cluster ranging from K = 1 to K = 3, ten independent runs with 100 000 Markov Chain Monte Carlo repetitions each and a burn-in period of 50 000 were performed. The analyses were based on the admixture model with correlated allele frequencies. As only three populations are considered in the present study, the best estimate of distinct genetic clusters was determined based on the highest mean log likelihood [ln P(D)].

3 Results

3.1 Genetic Diversity at the Population Level

Microsatellite study. A general excess of homozygotes for most allele size classes was observed at microsatellite loci Bo.G182 and Bo.F394 in the LIN population and at locus L022 at the ZIEL locality, which could suggest the presence of null alleles. Very strong support for the presence of null alleles would be provided by the presence of individuals with a permanent lack of amplification at some loci (null allele homozygotes). There were no such individuals in our material; therefore, we decided to consider all loci in further analyses, taking into account the likely presence of null alleles in the estimation of F_{ST}. No stutter peaks and large allele dropouts were found.

All loci were recognised as polymorphic in the total sample; however, one locus at the LIN site and two loci at IZER were monomorphic. We screened a total of 99 alleles, 2–16 alleles per locus, with an average of nine alleles per locus (Table 2). The highest mean number of alleles per population was observed at the LIN reserve (A = 6.09), whereas this measure was much lower in the mountain populations: A = 3.36 at IZER and A = 4.27 at ZIEL (Table 3). The average number of effective alleles was also the highest in the LIN population (A_E = 4.13), and the lowest at the IZER locality (A_E = 2.43). We scored 62 private alleles: 33 at the LIN, 13 at IZER and 16 at ZIEL. Forty of the 62 private alleles reached a frequency above 0.05 and thus should not be considered rare. The observed level of heterozygosity over all populations was moderate (mean H_O = 0.556; range 0.529–0.610) and almost equal to the expected heterozygosity (mean H_E = 0.562; range 0.507–0.641) (Table 3).

We found significant LD between L5.4 and L1.10, L2.7 and L13.1 at the IZER, between L1.10 and L2.7, L13.1 and L5.4 at the ZIEL and between L1.10 and L2.7, L13.1 and L5.4 in the entire data set. No genotypic disequilib-

Table 2. Genetic characteristic of the molecular markers used in the studies of *B. nana* populations in Poland. N – number of alleles per locus, A_E – mean effective number of alleles per locus, N_{PA} – number of private alleles, pb – size range of PCR product, H_O – observed heterozygosity, H_E – expected heterozygosity, * – sizes (bp) of RFLP fragments.

Microsatellite locus	N	A_E	N_{PA}	pb	H_O	H_E
L1.10	11	4.17	6	158–188	0.812	0.760
L2.7	15	4.08	12	159–207	0.849	0.755
L13.1	9	3.68	6	78–110	0.753	0.728
L5.4	4	2.04	2	238–246	0.440	0.509
L5.1	16	4.27	8	283–337	0.981	0.766
Bo.G182	12	2.22	10	117–155	0.419	0.550
Bo.F394	7	2.20	4	138–172	0.418	0.545
L3.1	3	1.12	2	211–217	0.118	0.104
L2.3	2	1.24	0	196–198	0.157	0.196
L021	15	3.73	10	172–216	0.660	0.732
L022	5	2.14	2	171–181	0.540	0.532
Total	99	30.89	62		0.556	0.562

cpDNA	TF-*Taq*I*	TF-*Hinf*I*	AS-*Taq*I*	CD-*Taq*I*	CD-*Hinf*I*
Haplotype I	540,490,340, 320,190	360,320,260,230, 150,120,110,80,70,60	1000,580,490,350, 280,260,140,120	600,450,390,360, 325,230,60	330,290,240,190, 140,115,85,70
Haplotype II	540,490,340, 320,190	360,320,260,230, 150,120,110,80,70,60	1000,580,490,380, 280,260,140,120	600,430,390,360, 325,230,60	330,260,240,190, 140,115,85,70

Table 3. Descriptive statistics of nuclear microsatellite loci in *B. nana* populations in Poland. P – proportion of polymorphic loci, A – mean number of alleles per locus, A_E – mean effective number of alleles per locus, N_{PA} – number of private alleles, H_O – observed heterozygosity, H_E – expected heterozygosity, p(HWE) – p value for HWE, F_{IS} – inbreeding coefficient.

Population	P (%)	A	A_E	N_{PA}	H_O	H_E	p(HWE)	F_{IS}
LIN	90.9	6.09	4.13	33	0.610	0.641	0.0512	0.050
IZER	81.8	3.36	2.43	13	0.530	0.507	0.0001	–0.047
ZIEL	100.0	4.27	3.02	16	0.529	0.537	0.0001	0.014
Total		4.57	2.81	62	0.556	0.562	<0.0001	0.008

ria were found at the LIN locality, which was the only population where we observed female catkins. The IZER and ZIEL reserves deviated significantly from HWE (Table 3). Lack of HWE was caused by the loci Bo.G182, L022 and L5.1 in the IZER population and L13.1 and L5.1 at ZIEL. The most common form of deviation from Hardy-Weinberg proportions was an excess of heterozygotes. None of the studied populations exhibited signs of inbreeding, and the average inbreeding coefficient was very low and statistically nonsignificant (F_{IS} = 0.008). The Wilcoxon test implemented in BOTTLENECK software detected recent reductions in population sizes under the infinite allele model (IAM) at the LIN and IZER localities (Table 4). A recent genetic bottleneck was confirmed for IZER under the TPM model. According to the SMM, none of the studied populations experienced a recent bottleneck. The M-ratios were significantly reduced for all studied localities (Table 4).

cpDNA study. Two haplotypes were detected from all of the sampled material (Table 1, 2). All populations were monomorphic; haplotype I was found at the LIN and ZIEL localities (total frequency of 65.4%) and haplotype II (34.6%) was fixed at the IZER site. Total cpDNA haplotype diversity (H_E) was equal to 0.462.

Table 4. Results based on heterozygosity excess and M-ratio tests. * statistically significant value.

	Bottleneck			M-ratio	
	IAM	TPM	SMM	M value	p
LIN	0.00098*	0.18750	0.83887	0.44497*	0.0000
IZER	0.00195*	0.02441*	0.32617	0.54619*	0.0054
ZIEL	0.10303	0.35010	0.81738	0.56442*	0.0075

3.2 Genetic Differentiation among Populations

Microsatellite study. Values of F_{ST} were calculated after excluding the L1.10 locus, which exhibited significant linkage disequilibrium with three other loci in the entire data set. The value of F_{ST} was high and statistically significant among all studied populations (F_{ST}=0.185; 95% CI: 0.115–0.272). High values of genetic differentiation were also observed between all pairs of populations. The mountain populations IZER and ZIEL were the most strongly differentiated from one another (F_{ST}=0.229; 95% CI: 0.143–0.346) (Table 5). STRUCTURE analysis estimated the highest mean log likelihood at K=3 [ln P(D) (–1334.0)], indicating that each population of *B. nana* constitutes a distinct genetic cluster. The estimation of the effective number of migrants (Nm) among populations using the private allele method showed a mean frequency of private alleles of 0.143 with a resulting Nm of 0.35.

cpDNA study. Pairwise comparisons of population differentiation demonstrated only two values – the minimum for localities having the same cpDNA haplotypes and the maximum for localities where different haplotypes were fixed (Table 5). As the mountain populations of *B. nana* are geographically the closest but, at the same time, represented by different haplotypes, it is clear that seed flow between Polish populations of the species is absent.

Table 5. Pairwise population differentiation estimates with microsatellites (above the diagonal) and cpDNA haplotypes (below the diagonal) data sets. * statistically significant value.

	LIN	IZER	ZIEL
LIN	–	0.185*	0.139*
IZER	1.000*	–	0.229*
ZIEL	0.000	1.000*	–

4 Discussion

4.1 Genetic Diversity of *B. nana* in Poland

Genetic variability in the Polish populations of *B. nana* seems to be lowered compared to the widespread plants, as the average heterozygosity of the species is consistent with that calculated for narrowly distributed taxa (H_E=0.53, H_O=0.52; Nybom 2004). A closer inspection of our data makes it apparent that the populations of *B. nana* are not genetically eroded to the same extent. In the largest LIN population, we observed distinctly higher values of the genetic parameters compared to those from the much smaller mountain localities, IZER and ZIEL. Considerable genetic variation based on RAPD markers in the LIN population was previously described by Dąbrowska et al. (2006). Nuclear microsatellite depletion in the IZER and ZIEL samples could be a consequence of their limited sizes. In general, small populations are subjected to strong genetic drift that, by chance, fixes some alleles and eliminates others. Indeed, we observed high frequencies (>0.05) for 40 out of the 62 private alleles. The most extreme example was private allele 133 at locus Bo.G182, which reached a frequency of 0.912 in the ZIEL population. Hence, genetic drift is most likely responsible for significant departures from HWE in the mountain localities.

The bottleneck is another factor that could negatively influence the genetic structure of the studied populations. According to the IAM, the LIN and IZER localities underwent reductions in numbers that resulted in an excess of het-

erozygotes for nuclear microsatellites. TPM also detected a bottleneck in the IZER sample. In turn, the statistically significant values for the M-ratio revealed that all of the study populations were reduced in the past. As the recovery time of the M-ratio is longer compared to the heterozygosity excess test implemented in BOTTLENECK, the low value of M reflects older and more severe reductions in numbers in the study populations (Garza and Williamson 2001, Williamson-Natesan 2005). Thus, the low M-ratio values in all of the study populations are likely to be a consequence of genetic decline during postglacial recolonisation. The significant population reductions at the LIN and IZER localities that were revealed by the BOTTLENECK program may have resulted from contemporary processes, e.g., the overgrowth of the surface of the mires by other plants. In 1978–1996, an increase in the contribution of *Betula pubescens* (by approximately 100%) restricted open bog communities with *B. nana* by 22% in the LIN reserve (Ejankowski and Kunz 2006). In the IZER population, the dwarf birch is displaced by *P. mugo* and *M. caerulea* (Matuła et al. 2000, Kruszelnicki and Fabiszewski 2001), which has most likely led to a considerable reduction in population over the last ten years.

The genetic structure of the study populations might also be influenced by selection pressure. Lowered values of genetic diversity parameters in the marginal populations of *B. nana* located in the Arctic archipelago of Svalbard were explained as a consequence of selection acting through climatic conditions (Alsos et al. 2002). We think that the Polish populations of the dwarf birch could also be under a selection pressure because they occupy marginal areas and suffer from the overgrowth by *P. mugo* and *M. caerulea*. An evidence of selection acting in the Polish samples could be a lack of sexual reproduction, expressed as the absence of female catkins, in the Sudety Mts. populations in 2011. It was suggested that habitat loss and fragmentation have forced plant populations to allocate more resources to vegetative than generative reproduction, as it was shown in endangered shrub *Haloragodendron lucasii* (Sydes and Peakall 1998). The reproductive capacity of the seaweed *Fucus serratus* was also dramatically reduced in marginal populations relative to central ones (Viejo et al. 2011). At the IZER locality, *B. nana* was confirmed to be flowering and bearing fruit in the 1990s (Matuła et al. 2000, Kruszelnicki and Fabiszewski 2001). Birches usually produce varying quantities of seeds every year (Atkinson 1992); thus, the lack of seeds in the mountain populations of the dwarf birch is distressing. To confirm whether the absence of seeds in 2011 was an accidental or long-term phenomenon and identify what factors were responsible for it, monitoring will be necessary over the coming years.

Gene flow is an important force for the maintenance of genetic diversity. Slatkin (1987) suggested that one immigrant every second generation or one interpopulation mating per generation prevented an increase in differentiation among populations. In our study, the private allele frequency method indicated that the exchange of genes between the populations of *B. nana* was highly restricted ($Nm = 0.35$), resulting in considerable and statistically significant pairwise population differentiation. This is not surprising because all populations are geographically isolated from one another, and in such a situation it is difficult to imagine regular gene flow due to the limited height of *B. nana*. The analysis of cpDNA variation supports the idea that gene flow via seeds does not exist because the populations nearest to one another, IZER and ZIEL, are characterised by different haplotypes. A lack of gene exchange was also revealed by the analyses conducted with STRUCTURE software. These analyses confirmed that each population of the dwarf birch constituted an independent unit.

The most striking result of our study was the considerable contribution of private alleles in the gene pools of all of the Polish populations of *B. nana*. Previously studied isolated marginal populations of the endangered species *Betula humilis* comprised only a few such alleles (Jadwiszczak et al. 2011a, b). In our opinion, the considerable contributions of private alleles in all of the studied *B. nana* localities are an effect of very restricted gene flow. Slatkin (1985) stated that a high frequency of private alleles indicated that gene pools might evolve independently from one another to some extent.

4.2 Glacial and Postglacial History of *B. nana* in Poland

The border of the maximum extent of the Weichselian ice sheet in Poland passed through Gubin, Zielona Góra, Leszno, Konin, Płock, Nidzica, Grajewo and continued further east in the direction of Grodno in Belarus (Fig. 1). At the forefield of the ice sheet the broad periglacial zone was formed, which created favourable conditions for *B. nana* during the entirety of the most recent glaciation. Indeed, palynological investigations have revealed the continuous occurrence of the birches belonging to the *Nanae* section in the refugia dated to the Weichselian Pleniglacial in the northern Carpathians and their foreland (Dobra, Nowa Huta, Brzeźnica and Smerek sites; see Ralska-Jasiewiczowa et al. 2004 and references therein).

B. nana is an indicator of low temperatures and, thus was more widespread during stadials (Oldest Dryas, Older Dryas and Younger Dryas) than interstadials (Bølling, Allerød) of the Late Glacial. Remains dated to the stadials are found throughout Poland: Gościąż Lake (Ralska-Jasiewiczowa et al. 1998), Lednica Lake (Tobolski 1998), Pomieczyno (Marek 1991), Niechorze (Ralska-Jasiewiczowa and Rzętkowska 1987), Durne Bagno (Bałaga 2007), the Knyszyńska Forest (Kupryjanowicz 2000, Drzymulska 2006), the Carpathians (Margielewski et al. 2003) and Wieluń (Nita and Szymczyk 2010). Dwarf shrubs have appeared at the Linje mire in the Younger Dryas (Noryśkiewicz 2005, Kloss 2007) and are also present currently.

In the Preboreal Period of the early Holocene, the dwarf birch occurred in north-eastern (Miłkowskie Lake; Wacnik 2009) and central Poland (Długie Bagno mire; Kloss 2007). Since the Boreal period, *B. nana* is connected persistently with mires in the Sudety Mts. – Zieleniec (Madeyska 2005, Kloss 2007) and Torfowiska Doliny Izery (Popowski 2005).

The distribution of historic populations of the dwarf birch in Poland indicates that this species was confined to the southern part of the country at the Last Glacial Maximum; however, it is impossible to determine which cpDNA haplotypes were present in that region because all Weichselian Pleniglacial populations are extinct. Two most frequent birch haplotypes arose likely before the last glaciation (Palmé et al. 2004, Jadwiszczak et al. 2012), thus, at least one of them could have survived in the Carpathians. Another problem is that neither the palaeobotanical data nor cpDNA haplotypes allow an inference of the directions of *B. nana* recolonisation after the LGM. In general, central Europe was populated by birches from the eastern and western directions from refugia located at higher latitudes (Palmé et al. 2003, 2004, Maliouchenko et al. 2007, Jadwiszczak et al. 2012). The same phenomenon occurred in Scandinavia (Palmé et al. 2003, 2004, Maliouchenko et al. 2007). We recorded cpDNA haplotypes I and II in the studied populations of *B. nana*, the same that predominate in *B. humilis* populations (Jadwiszczak et al. 2012). Most likely, these haplotypes correspond to haplotypes A and C described in the Scandinavian distribution of *B. nana* and the tree birches *B. pendula* and *B. pubescens*; however, a direct comparison of haplotypes is not possible due to the lack of information on fragment sizes in the papers of Palmé et al. (2003, 2004) and Maliouchenko et al. (2007). The presence of two haplotypes only in the Polish populations can be explained in the two ways. First, after the Holocene warming Poland was recolonised from two distinct refugia. One refugium could exist in the Carpathians and their northern foreland, as has been suggested based on the palynological record (Ralska-Jasiewiczowa et al. 2004). Second explanation is the fixation of single haplotypes in the studied populations could result from the genetic drift acting on the relict samples. To answer this question a genetic analysis of remnant localities of *B. nana* from the Alps, Germany and Czech Republic as well as from Belarus and the Baltic countries is necessary.

4.3 Conservation Strategy

We hypothesise that all of the unfavourable processes influencing the genetic structure of the dwarf birch populations are consequences of their very limited sizes. All Polish localities are spread over an area less than 10 km^2. For such rare and local species that are common in other parts of the Earth, Crain and White (2011) proposed to employ the novel category L1 (locally critically imperilled) followed by the IUCN category EN

(endangered) – ENL1. Especially vulnerable to extinction are the dwarf birch populations situated in the Sudety Mts., where we observed considerable reductions in the average number of alleles per population compared to *B. humilis,* the endangered species in Poland (Jadwiszczak et al. 2011a, b). A decrease in allelic variation where expected heterozygosity still remains relatively high indicates that a population has recently undergone a substantial reduction (Nei et al. 1975).

Our analyses have confirmed that the dwarf birch in Poland is threatened. This strong expression is also based on the linkage disequilibria detected between a few microsatellite loci at the ZIEL and IZER localities as well as in the total sample. In the focal species, the considered microsatellites belong to different linkage groups (Pekkinen et al. 2005); hence, the increase in LD in the study populations can be an effect of small population size, genetic isolation between populations, a low recombination rate or natural selection (Gupta et al. 2005). However, in contrast with the *B. nana* localities inhabiting the Svalbard archipelago (Alsos et al. 2002), we discovered many genetically distinct individuals in every population. This finding, together with the very high contribution of private alleles and the distribution of cpDNA haplotypes, strongly suggests that each Polish population of the dwarf birch is valuable for protection. The restoration of natural gene flow among Polish localities is impossible. In turn, a transplantation of plants among Polish populations is highly inadvisable. First, the dwarf birches from the LIN and ZIEL reserves belong likely to another phylogenetic group than the IZER sample. Second, the LIN and ZIEL populations have been evolving independently for thousands of years; hence, they could be adapted to the local environments. For the above reasons, the only treatment for enhancing genetic variation is to provide suitable conditions for sexual reproduction in the study populations. We suggest that an improvement in *B. nana* habitat conditions in the Sudety Mts. could be achieved by removing *P. mugo. P. mugo* is also under strict protection in Poland, but it is not as rare as the dwarf birch and is placed within the LC (Least Concern) category of the IUCN. *P. mugo* has low habitat requirements; it grows well on different kinds of soils: acidic and alkaline, dry and wet, shallow and deep. In our opinion, an expansion of *P. mugo* is a result of: drainage conducted in the area of mires several decades ago, a deterioration of habitat conditions of other plants, as a consequence of the ecological catastrophe in the western part of the Sudety Mts. in 1977–1985, and the cessation of *P. mugo* removing from the expanded area of the reserves. The Linje mire was also drained in the past which caused an expansion of *B. pubescens.* The existence of *B. nana* was clearly improved due to anthropogenic deforestation conducted in the 1990's (Kruszelnicki and Fabiszewski 2001). However, this manipulation had only a short-term result because regeneration of *B. pubescens* is still possible in the current habitat conditions (Ejankowski and Kunz 2006).

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