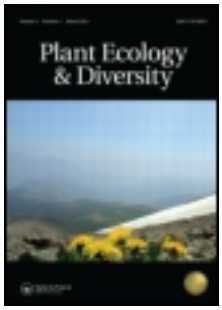

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The admixture zone of *Betula humilis* Schrk. phylogenetic lineages follows the eastern central European suture zone

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Background: Contemporary genetic variation across species ranges reflects Pleistocene climatic changes. The highest genetic diversity is usually found within areas of known or presumed glacial refugia and within the admixture zones of phylogenetic lineages.
Aims: The aim of our study was to evaluate the genetic diversity in the populations of *Betula humilis*, an endangered species, to distinguish between the refugial and suture zone hypotheses.
Methods: We used the PCR-RFLP method to analyse the chloroplast DNA variation in populations of *B. humilis*, distributed across north-eastern Poland, western Belarus and Latvia. Rarity index (DW) was calculated for each population based on 'frequency-down-weighted marker values'. We tested phylogeographic structure by using the Permut software.
Results: The area studied was phylogeographically structured; DW values were low.
Conclusions: Based on the low DW values, we rejected the hypothesis of a periglacial refugium in north-eastern Poland and Belarus. Most likely, the substantial genetic diversity in the area under study is a consequence of the mixing of phylogenetic lineages derived from distinct glacial refugia. The admixture zone of *B. humilis* follows the eastern central European suture zone.

Keywords: *Betula humilis*; chloroplast DNA; conservation genetics; PCR-RFLP method; rarity index; suture zone

Introduction

Trees and shrubs of the genus *Betula* represent characteristic elements of the boreal and temperate flora of the northern hemisphere. In Europe, four birch species are distinguished: *Betula pendula* Roth, *B. pubescens* Ehrh., *B. humilis* Schrk. and *B. nana* L. *B. pendula* and *B. pubescens* are tall trees and are sympatrically distributed throughout almost the entire continent. The shrub birch, *B. humilis*, and the dwarf birch, *B. nana*, are endangered species (EN category of the International Union for Conservation of Nature) in the western and central parts of Europe (Kruszelnicki and Fabiszewski 2001; Załuski et al. 2001).

The effective conservation of endangered species is not possible without information on the levels and distribution of genetic variation within and among populations. A large body of evidence suggests that contemporary patterns of genetic diversity across plant species distributions result from vegetation dynamics forced by climate changes during the Pleistocene (Petit et al. 2003; Hu et al. 2009; Avise 2009). Most European plants survived the Last Glacial Maximum (LGM) in well-defined refugia, situated in the southern parts of the continent (Taberlet et al. 1998; Hewitt 1999; Petit et al. 2003). However, increasing amounts of palaeobotanical and phylogeographical evidence strongly suggest that cold-tolerant species could also have occurred in 'microrefugia' located at higher latitudes (Birks and Willis 2008; Paun et al. 2008; Daneck et al. 2011; Jadwiszczak 2012). Putative populations of glacial refugia usually retain high levels of genetic variation. These high levels make them worthy of protection (Hampe and Petit 2005). After the retreat of the Scandinavian glacier, refugial populations expanded northwards into the ice-free areas, gradually losing genetic variation due to the 'founder effect'. However, not all newly recolonised territories show a low level of genetic variation. As a consequence of Holocene range expansion, waves of migration originating from distinct glacial refugia met and mixed to form contact or hybrid zones. The mixing of phylogenetic lineages may result in even greater genetic variation than that observed in refugial populations (Petit et al. 2003); thus, the study and conservation of populations situated within admixture zones often aid the preservation of substantial genetic resources for the species of interest. These measures are especially important for threatened taxa because a sufficient amount of genetic diversity is crucial for their survival under climatic and anthropogenic changes in the environment.

Investigations using nuclear microsatellites and restriction enzyme analysis of PCR fragments (PCR-RFLP) have demonstrated substantial genetic variation in endangered *B. humilis* populations in north-eastern Poland and central Belarus (Jadwiszczak et al. 2011a, b, 2012). Two hypotheses

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have been proposed to explain this phenomenon: the existence of a periglacial refugium and an admixture zone, formed from distinct phylogenetic lineages. The results of phylogeographical studies strongly suggest that *Betula* species could have survived the LGM at higher latitudes (Palmé et al. 2003; Maliouchenko et al. 2007; Jadwiszczak et al. 2012). This hypothesis is supported by the occurrence of *B. humilis* pollen and macrofossils dated to the Middle Weichselian Pleniglacial (59,000–28,000 years ago) in Ukraine (Stachowicz-Rybka et al. 2009), Hungary (Medzihradszky and Bajzáth 1998) and Poland (Velichkevich and Mamakowa 1999). The species was also well established in northern Germany during the Weichselian (Freund et al. 2001). However, the low frequency of private cpDNA haplotypes (Jadwiszczak et al. 2012), the low contribution of private alleles of nuclear microsatellites, and the high within-population diversity and low inter-population differentiation of nuclear microsatellites in the area under consideration (Jadwiszczak et al. 2011a) suggest that a periglacial refugium for *B. humilis* was, most likely, not present in north-eastern Poland. Rather, an analysis of the relationship between allelic diversity and a measure of divergence of cpDNA markers implied that the substantial genetic variation found in the north-eastern Polish and central Belarusian populations of *B. humilis* might have resulted from an admixture of phylogenetic lineages (Jadwiszczak et al. 2012). Previous investigations on three populations located in periglacial areas in north-eastern Poland were inconclusive in determining if the high genetic diversity of the species reflects a heritage derived from a periglacial isolate or results from the mixing of different phylogenetic lineages. Therefore we conducted a wider study. The specific objectives of the study were to: (1) evaluate the haplotype diversity occurring in additional localities of *B. humilis* from north-eastern Poland, (2) identify cpDNA variation in western Belarus and Latvia and (3) compare the genetic resources represented by rare haplotypes in *B. humilis* populations with those from plants having known glacial refugia.

Material and methods

In all, 105 individuals of *B. humilis* were sampled from eight populations in Poland, Latvia and Belarus (Table 1; Figure 1). Five to 17 individuals per population were sampled randomly at a distance of at least 10 m from each other to avoid collecting samples from the same genet. Three young, healthy leaves were sampled per individual and immediately dried in silica gel. The dried leaves were ground with a TissueLyser LT bead mill (Qiagen). The total DNA was then extracted using an AX Plant kit (A&A Biotechnology) according to the manufacturer's instructions.

The PCR-RFLP method was used to analyse cpDNA diversity. Three non-coding cpDNA regions (*trn*T-*trn*F, *psa*A-*trn*S, *trn*C-*trn*D) were screened for possible variation using the primer pairs described by Taberlet et al. (1991) and Demesure et al. (1995). PCR amplifications were carried out in a total volume of 10 µl of a mixture containing 4.0 µl of diluted DNA (DNA concentration was 16.2–197.5 ng μl^{-1}), 3.4 µl of Multiplex PCR Master Mix (Qiagen), 2.0 µl of RNase-free water (Qiagen) and 0.6 µl of primer mixture (0.2 µM of each primer). Touchdown PCR reactions of all cpDNA fragments were performed with a TProfessional thermocycler (Biometra). The profiles of the touchdown PCR reactions were obtained according to Jadwiszczak et al. (2012). The amplified TF and CD fragments were then digested using the FastDigest restriction enzymes *Taq*I and *Hinf*I (Fermentas). The AS fragment was digested using *Taq*I only. The digestion mixtures and restriction procedures are described in Jadwiszczak et al. (2012). PCR-RFLP fragments were separated by horizontal electrophoresis on 1.5% agarose gels in 1×TBE buffer and stained with ethidium bromide. Fragment sizes were determined by comparison with a 50-bp DNA ladder size standard (O'Range Ruler, Fermentas) that was run on each gel. The results were visualised and photographed with a UV camera (BioRad).

Gene diversity (H_E) within each population was calculated with Arlequin version 3.5 (Excoffier and Lischer 2010). Diversity and differentiation parameters [h_S – the average intra-population gene diversity, h_T – the total diversity, G_{ST} – the differentiation for unordered alleles (the frequency of particular haplotype is considered only) and v_S, v_T and N_{ST}, the corresponding parameters for ordered alleles (both the frequency of haplotypes and number of mutation between particular haplotypes are taken into account)] were estimated according to Pons and Petit (1996) using Permut software (http://www.

Table 1. Locations, codes and sample sizes of the *Betula humilis* populations studied in Poland, Belarus and Latvia.

Country	Population name	Code	Coordinates		Sample size
			Latitude	Longitude	
Poland	Sojczyn Grądowy	SOJ	N 53°34′	E 22°37′	17
	Stara Kamienna	SK	N 53°42′	E 23°18′	13
	Grzędy	GRZ	N 53°36′	E 22°52′	17
	Maliszewskie Lake	MAL	N 53°10′	E 22°30′	12
Belarus	'Dikoe' swamp	DZI	N 52°44′	E 24°17′	16
	Koldyčeskoe Lake	KOL	N 53°16′	E 26°03′	11
	Germaniški	GER	N 54°07′	E 25°21′	14
Latvia	Limbažu Lielezers Lake	LL	N 57°28′	E 24°41′	5

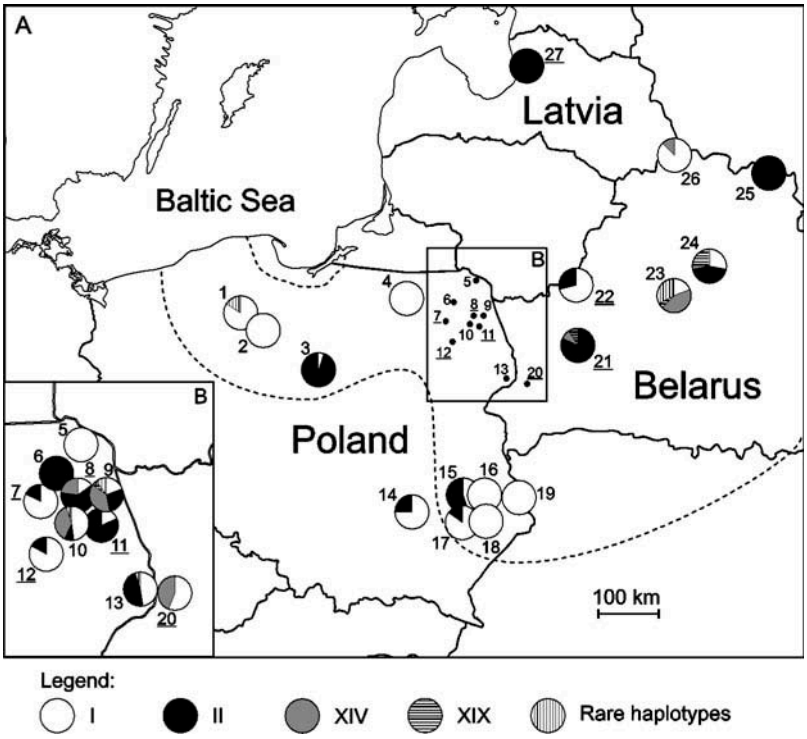

Figure 1. Distribution of *Betula humilis* haplotypes: (A) all populations studied in Poland, Belarus and Latvia, (B) enlarged area of north-eastern Poland and western Belarus. Population numbers are according to Table 2. Underlined numbers correspond to samples collected in the present study. Populations 1–6, 9–10, 13–19, 23–26 after Jadwiszczak et al. (2012). Dashed lines indicate the range o *B. humilis*.

pierroton.inra.fr/genetics/labo/Software). Calculations of diversity and differentiation parameters were conducted for the eight localities considered in the present study as well as for a total sample of 18 populations located in a presumed suture zone, i.e. 10 localities from north-eastern Poland (JEZ, MB, ROS, SOJ, SK, SUS, CB, GRZ, MAL, BIA), seven Belarusian localities (DZI, KOL, GER, SLU, BZ, IS, OS) and one population from Latvia (LL) (Table 2). Data from the JEZ, MB, ROS, SUS, CB, BIA, SLU, BZ, IS and OS populations are after Jadwiszczak et al. (2012). Permut was also used to identify potential phylogeographic structure (Pons and Petit 1996; Burban et al. 1999). Phylogeographic structure is indicated by the relationship $N_{ST} > G_{ST}$, which implies that more closely related haplotypes are found in the same population more frequently than unrelated haplotypes (Pons and Petit 1996). The significance of the relationship between N_{ST} and G_{ST} relationships was tested using 10000 permutations. The permutation test is significant if fewer than 5% of the permuted values are greater than the observed N_{ST} (Pons and Petit 1996).

For each population considered in the present study, as well as for all samples from our previous research (Jadwiszczak et al. 2012), a rarity index (a measure of population genetic divergence) was manually calculated based on 'frequency-down-weighted marker values' (DW) (Schönswetter and Tribsch 2005; Paun et al. 2008; Daneck et al. 2011). In our study, the term 'marker' is related to a particular haplotype because each haplotype represents a unique combination of PCR-RFLP fragments (for haplotype description see Jadwiszczak et al. 2012). The values of DW were estimated by dividing the number of each haplotype in the population by the number of occurrences of this haplotype in the total sample of 470 individuals (Table 2). The values were then summed to obtain the DW index for the population (Paun et al. 2008; Daneck et al. 2011).

Results

Four PCR-RFLP haplotypes, namely, I, II, XIV and XIX, were found in the eight populations studied (Table 2). These common haplotypes have been previously described by Jadwiszczak et al. (2012). Haplotype I was observed in seven populations (frequency of 45.7%) but was absent from KOL, whereas haplotype II (42.9%) was only absent from DZI. Haplotype XIV was found in SK, DZI and KOL (10.5%), and haplotype XIX occurred once, in KOL (0.9%). Three haplotypes were observed in the SK and KOL populations, two in SOJ, GRZ, MAL, DZI and GER and one in the Latvian LL sample. The observed gene diversity ranged from $H_E = 0.00$ (LL) to 0.59 (SK).

The total diversities estimated for the eight populations of *B. humilis* were almost the same for unordered and ordered alleles, $h_T = 0.620$ and $v_T = 0.623$, respectively (Table 3). The analogous values for the total sample of 18 populations from north-eastern Poland, Belarus and Latvia were slightly higher, $h_T = 0.665$ and $v_T = 0.669$,

Table 2. Number of individuals with particular cpDNA haplotypes, gene diversities (H_E) and within-population rarities of haplotypes (DW) in *Betula humilis* populations studied in Poland, Belarus and Latvia. Population numbers correspond to Figure 1. *except for DW, data after Jadwiszczak et al. (2012); RH, rare haplotypes (haplotypes III, IV, V, VI, X, XI, XI, XV, XVI, XVII, XVIII, XX, XXI; after Jadwiszczak et al. 2012).

			Haplotypes						
Country	Population number	Population code	I	II	XIV	XIX	RH	H_E	DW
Poland	1	JM*	16	-	-	-	3	0.30	3.062
	2	LS*	20	-	-	-	-	0.00	0.078
	3	TM*	1	19	-	-	-	0.10	0.132
	4	JEZ*	19	-	-	-	-	0.00	0.074
	5	MB*	19	-	-	-	-	0.00	0.074
	6	ROS*	-	16	-	-	3	0.30	3.107
	7	SOJ	14	3	-	-	-	0.31	0.074
	8	SK	2	8	3	-	-	0.59	0.141
	9	SUS*	4	6	8	1	2	0.77	2.377
	10	CB*	10	2	8	-	1	0.65	1.263
	11	GRZ	3	14	-	-	-	0.31	0.106
	12	MAL	10	2	-	-	-	0.30	0.052
	13	BIA*	10	10	-	-	1	0.57	1.106
	14	PAK*	6	2	-	-	-	0.43	0.036
	15	MO*	9	11	-	-	-	0.52	0.109
	16	KB*	20	-	-	-	-	0.00	0.078
	17	UU*	17	3	-	-	-	0.27	0.086
	18	BB*	20	-	-	-	-	0.00	0.078
	19	TS*	20	-	-	-	-	0.00	0.078
Belarus	20	DZI	9	-	7	-	-	0.52	0.219
	21	KOL	-	9	1	1	-	0.34	0.197
	22	GER	10	4	-	-	-	0.44	0.066
	23	SLU*	4	-	9	1	7	0.75	3.363
	24	BZ*	7	11	1	6	-	0.69	0.794
	25	IS*	-	24	-	-	-	0.00	0.161
	26	OS*	7	-	1	-	-	0.25	0.053
Latvia	27	LL	-	5	-	-	-	0.00	0.034
		Total	257	149	38	9	17		

Table 3. Estimated parameters of cpDNA diversity for unordered and ordered alleles in *Betula humilis* populations. h_S and v_S, average intra-population diversity, h_T and v_T, total diversity, G_{ST} and N_{ST}, differentiation among populations, *, statistically significant difference ($P < 0.05$) between G_{ST} and N_{ST}.

	Levels of population subdivision					
	Unordered alleles			Ordered alleles		
Populations	h_S	h_T	G_{ST}	v_S	v_T	N_{ST}
SOJ, SK, GRZ, MAL, DZI, KOL, GER, LL[a]	0.353±0.063	0.620±0.046	0.431±0.112	0.328±0.070	0.623±0.058	0.474±0.131
JEZ, MB, ROS, SOJ, SK, SUS, CB, GRZ, MAL, BIA, DZI, KOL, GER, SLU, BZ, IS, OS, LL[b]	0.392±0.062	0.665±0.039	0.410±0.079	0.334±0.061	0.669±0.049	0.500±0.096*

[a], present study; [b], all populations located in a presumed admixture zone. Data from JEZ, MB, ROS, SUS, CB, BIA, SLU, BZ, IS and OS after Jadwiszczak et al. (2012).

respectively. In both analyses, the inter-population differences were large, G_{ST} = 0.431 for eight populations and G_{ST} = 0.410 for 18 populations. Of 10000 permutations performed for eight populations, 2457 values (24.57%) were greater than the observed N_{ST}. This statistically non-significant result means that the sampled populations were not phylogeographically structured. However, the difference between N_{ST} and G_{ST} was significant when the 18 localities were considered (only 1.30% of the permuted values were greater than the observed N_{ST}).

Of the 27 populations of *B. humilis* in which cpDNA variation has been investigated to date, the value of the rarity index, DW, was the lowest in LL and the highest in SLU, 0.034 and 3.363, respectively (Table 2). High DW values were also observed in the ROS (3.107) and SUS (2.377) populations, both from north-eastern Poland, and

in JM (3.062), from northern Poland. Among the eight populations sampled for the purposes of the present study, the rarity index reached its highest values in the Belarusian localities DZI (0.219) and KOL (0.197).

Discussion

The rarity index, DW, is a good indicator of a putative glacial refugium. The values of this index are higher in the populations with substantial numbers of rare markers. Such markers are expected to accumulate in long-isolated populations as a result of mutation. It has been suggested that long-isolated populations are associated with areas of presumed glacial refugia (Daneck et al. 2011). In contrast, recently established populations exhibit low DW values, although, at the same time, they could present high genetic variation (Schönswetter and Tribsch 2005; Paun et al. 2008). This is because genetic variation results mainly from contemporary processes, while the contribution of rare markers reflects the history of population (Paun et al. 2008). Phylogeographic investigations of the temperate shrub *Lonicera nigra* using amplified fragment length polymorphism (AFLP) markers indicated high DW values in both the Pyrenees (DW = 7.19 and 7.63) and the Czech Republic (DW = 7.16 – 7.47). These values were assumed to reflect glacial survival in these areas (Daneck et al. 2011). Among 20 populations of the alpine vascular plant *Bupleurum stellatum* inhabiting presumed refugia in peripheral areas of the southern Alps, the highest value of DW calculated for AFLP markers was 18.16, the lowest 8.85 (Schönswetter and Tribsch 2005). In the *B. humilis* localities sampled, the DW indexes were substantially lower than those observed in *B. stellatum* and *L. nigra*. The highest values were noted in the SLU (3.363), ROS (3.107) and JM (3.062). It could be assumed that the low values of rarity indices in the *B. humilis* samples resulted from lower variation of chloroplast DNA markers compared with AFLP ones. However, the DW indices inferred from AFLP variation can be also low, as was described in the two arctic-alpine species *Sagina caespitosa* (DW = 0.96 – 3.57) and *Arenaria humifusa* (DW = 0.91 – 1.59; Westergaard et al. 2011) and in the polyploid *Linnaea borealis* subsp. *borealis* (DW = 0.81 – 3.22; Wróblewska 2013). The low values of the rarity index contradict the hypothesis that substantial genetic diversity of *B. humilis* in north-eastern Poland and Belarus could result from periglacial survival, especially considering that the ROS and JM populations are located within an area covered by the ice sheet during the LGM (Jadwiszczak et al. 2011a). Thus, the DW values in the *B. humilis* samples can result from the contemporary isolation of these populations rather than from periglacial survival.

Given the final rejection of the periglacial persistence hypothesis, the only explanation of the high genetic variation of *B. humilis* in north-eastern Poland and Belarus is the mixing of phylogenetic lineages. The sampled material strongly suggests that the admixture zone of *B. humilis* phylogenetic lineages extends continuously from northern Poland to at least central Belarus. This hypothesis is supported by the intermixing of PCR-RFLP haplotypes belonging to the different haplogroups. An analysis of cpDNA variation conducted previously in 19 populations of *B. humilis* situated in northern, north-eastern and south-eastern Poland and central Belarus revealed 17 haplotypes, which formed two haplogroups in the minimum spanning tree (Jadwiszczak et al. 2012). In samples collected from eight additional localities, i.e. SOJ, SK, GRZ, MAL from north-eastern Poland, DZI, KOL, GER from western Belarus and LL from Latvia, only four haplotypes were found, namely, haplotypes I, II, XIV and XIX. Haplotypes I and II are considered to represent the ancestral forms because they are the most common and widespread haplotypes found in *B. humilis* populations. Moreover, they assume a central position in the minimum spanning tree, furnishing additional evidence of their ancestry (Jadwiszczak et al. 2012). Two other haplotypes noted in the present study were XIV and XIX. Both of these haplotypes are related to haplotype I in the haplotype network. Haplotype I and/or its derivatives XIV and XIX coexist with haplotype II in all presently studied populations with the exception of the Belarusian DZI locality.

The admixture zone of *B. humilis* phylogenetic lineages is part of a suture zone that is located in eastern central Europe. A suture zone is a geographical region containing clusters of substantial numbers of phylogeographic breaks, contact zones and hybrid zones (Remington 1968; Taberlet et al. 1998). In Europe, five principal suture zones are found in the Alps, the Pyrenees, central Scandinavia, western central Europe and eastern central Europe (Taberlet et al. 1998; Hewitt 1999; Schmitt 2007). The eastern central European suture zone is formed by contact/hybrid zones of different phylogenetic lineages of the common hamster *Cricetus cricetus* (Banaszek et al. 2010), the steppe plant *Iris aphylla* (Wróblewska 2008) and the spring pasqueflower *Pulsatilla vernalis* (Ronikier et al. 2008), as well as by species of the genus *Betula* (Jadwiszczak 2012), among others. In general, the western and eastern central European suture zones have been considered to run along the western and eastern borders, respectively, of Germany. However, the lack of substantial topographic barriers in the North European Plain makes the particular components of both central European suture zones staggered relative to each other (Taberlet et al. 1998).

In the present investigation comprising eight populations of *B. humilis*, no phylogeographical structure was detected. However, a combined analysis of all populations from north-eastern Poland, Latvia, western and central Belarus gave significantly higher values of N_{ST} than of G_{ST}. This significant result suggests that the territory studied was recolonised from distinct glacial refugia (Fussi et al. 2010). Phylogeographical structure has previously been demonstrated for 19 populations of *B. humilis* (Jadwiszczak et al. 2012). Unfortunately, the palaeobotanical and phylogeographical data collected to date do not allow an unambiguous identification of the locations in

which *B. humilis* survived the LGM. The lack of isolation by distance found for both cpDNA markers and nuclear microsatellites implies that *B. humilis* could have persisted at higher latitudes during the LGM (Jadwiszczak et al. 2011a, 2012). This hypothesis is strengthened by fossil records found in Germany, Ukraine, southern Poland and Hungary and dating from the last glaciation (Medzihradszky and Bajzáth 1998; Velichkevich and Mamakowa 1999; Freund et al. 2001; Stachowicz-Rybka et al. 2009). Most likely, one glacial isolate of *B. humilis* was located in eastern Europe. During the entire Weichselian, the ecosystems of the Russian Plain persisted almost unchanged, and forest and forest-steppe plants are postulated to have survived within the Crimea, in the Trans-Carpathian regions, in the Caucasus, within the Central Russian Uplands, in the Donetsk Ridge and within the range of the Dnieper River (Markova et al. 2009). The eastern refugium hypothesis appears to be confirmed by the distribution of *B. humilis* cpDNA haplotypes across Poland and Belarus (Jadwiszczak et al. 2012). In view of the fossil record and present-day occurrence of the species, another glacial isolate is proposed to have been situated in the Carpathians or in western Europe. Presumed locations of the *B. humilis* glacial isolates mentioned above seems to be very likely; however, it is difficult to say whether these refugia were well defined or small rather. Macrofossil evidence suggests that *Betula* species could have survived the LGM in microrefugia scattered along the edge of the glacial areas (Willis et al. 2000; Palmé et al. 2003). This part of the *B. humilis* population history should be studied in the future.

The sampling conducted for this study did not indicate populations with substantial genetic variation other than those described previously (Jadwiszczak 2012). Six haplotypes were found in the most genetically diverse populations of *B. humilis* – SUS from north-eastern Poland and SLU from central Belarus. These results were associated with gene diversity (H_E) values of 0.77 and 0.75, respectively (Jadwiszczak et al. 2012). In the *B. humilis* localities sampled in the present study, at most three haplotypes were identified, and the highest H_E was found in the Polish sample SK (0.59). The SK population, like SUS, CB (H_E = 0.65) and GRZ (H_E = 0.31), is situated in Biebrza National Park. Of the Polish localities, SUS and CB are also characterised by the highest mean number of alleles per nuclear microsatellite locus (Jadwiszczak et al. 2011a).

Conclusions

As substantial genetic diversity is essential to the long-term survival of any population and species, it could be assumed that the most valuable localities for protection are *B. humilis* sites in the Biebrza river valley. It is widely accepted that patterns of genetic variation strongly depend on actual population size, reproductive performance and inter-population gene flow (Paun et al. 2008); thus, more detailed studies are needed in the future to establish to what extent each of these factors influences genetic variation of *B. humilis* in Biebrza National Park.

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