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Factors shaping genetic diversity of the shrub birch (*Betula humilis* Schrk.) in populations at the south-western margin of its range

PhD dissertation

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Contents

Abstract

The genetic diversity of plant populations results from the history of the species and contemporary factors, such as evolutionary processes, habitat conditions and species biology. As many peripheral populations are also ecologically marginal, their genetic diversity and demographic performance are particularly influenced by currently acting factors.

The shrub birch, *Betula humilis* Schrk., is a glacial relict in central and western Europe, and its continuous geographical range has a south-western boundary in Poland. The species grows mostly in fens and wet meadows, but being a poor competitor, it declines in dry habitats due to overgrowth by brushwood and forest plants. The number of *B. humilis* populations in Poland decreased approximately fourfold during the twentieth century. Although lowered genetic variation was noted in the smallest and most isolated stands, some populations of *B. humilis* located in north-eastern Poland still exhibited a high level of genetic diversity.

The primary aim of my PhD thesis was to define the factors that currently shape the genetic diversity of *B. humilis* populations at the south-western margin of its distribution. I investigated the efficiency of generative reproduction, the frequency of hybridisation with closely related tree congeners and the clonal architecture of the shrub birch populations under different habitat conditions. I expected that effective sexual reproduction and crossbreeding with common close relatives would positively impact genetic diversity. I also tested if aggregated clonal growth hindered crossing between genetically distinct individuals in *B. humilis* populations.

SSR (simple sequence repeat) marker analysis of the *B. humilis* population located in the Wizna mire, one of the largest declining fens in Poland, revealed that the shrub birch propagated exclusively by clonal growth (Chapter I). Only three genetically distinct specimens were found, which implied that generative reproduction was either absent or unsuccessful. The analysis of AFLP (amplified fragment length polymorphism) loci and sexual reproduction efficiency, conducted in eight marginal and three sub-central populations located in Poland and Belarus, revealed no significant correlations between genetic diversity and reproductive parameters, such as the number of flowers, seed mass and the number of germinated seeds (Chapter II). However, germination capacity was higher in sub-central

localities than in marginal stands as well as in wet habitats than in dry sites. This implies that unfavourable habitat conditions can be a significant factor leading to decrease in genetic diversity of shrub birch populations, especially in peripheral localities. Using the AFLP markers, analysis of the frequency of hybridisation between *B. humilis* and its close relatives *B. pendula* and *B. pubescens* was conducted in populations with different habitat conditions (Chapter III). Only three potential hybrids were detected in the shrub birch populations, although all of them were found in dry stands. This implies that low groundwater levels can intensify overgrowth by widespread birches and thus facilitate interspecific crossing through pollen swamping. Clonal structure analysis of the shrub birch was carried out in six populations by genotyping a total of 522 ramets at seven SSR loci (Chapter IV). The study showed that ramets belonging to the same clone were mostly aggregated. Nevertheless, the substantial clonal and genetic diversities implied that the clumped growth of the shrub birch clones did not prevent successful crosspollination.

These studies confirmed previous observations that the genetic diversity of marginal populations of the shrub birch was substantial. However, the primary factors responsible for the current maintenance of *B. humilis* populations are favourable habitat conditions, i.e., groundwater levels high enough to prevent the succession of competitive plants, which leads to shading, disrupts sexual reproduction of the light-demanding shrub birches, and causes population decline.

Streszczenie

Poziom zmienności genetycznej populacji roślin jest konsekwencją historii gatunku i czynników obecnie oddziałujących na populacje, do których zaliczają się zjawiska ewolucyjne, warunki środowiska czy cechy biologii gatunku. Populacje zlokalizowane na granicy obszaru występowania gatunku często są również ekologicznie marginalne, dlatego też ich zróżnicowanie genetyczne i demograficzne znajduje się pod szczególnie silnym wpływem powyższych czynników.

Brzoza niska, *Betula humilis* Schrk., jest reliktem glacjalnym w centralnej i zachodniej części Europy. W Polsce przebiega południowo-zachodnia granica jej zwartego zasięgu. Brzoza niska rośnie głównie na torfowiskach i podmokłych łąkach. Z uwagi na bardzo słabe zdolności konkurencyjne zanika w środowiskach suchych, gdzie jest wypierana przez gatunki zaroślowe i leśne. Liczba populacji *B. humilis* w Polsce zmniejszyła się około czterokrotnie w ciągu XX wieku. Na najmniejszych i najbardziej izolowanych stanowiskach brzozy niskiej stwierdzono obniżony poziom zmienności genetycznej, jednak niektóre populacje zlokalizowane w północno-wschodniej Polsce wykazały wysokie zróżnicowanie.

Głównym celem mojej pracy doktorskiej było określenie, jakie czynniki kształtują obecnie zmienność genetyczną w populacjach *B. humilis* na południowo-zachodnim krańcu zasięgu. W swoich badaniach sprawdziłam efektywność rozmnażania płciowego brzozy niskiej, częstość hybrydyzacji z blisko spokrewnionymi gatunkami drzewiastymi oraz strategię wzrostu klonalnego w populacjach zróżnicowanych pod kątem warunków środowiska. Oczekiwałam, że wysoka efektywność rozrodu i krzyżowanie z powszechnie występującymi gatunkami brzóz wpłynie pozytywnie na poziom zmienności genetycznej. Przetestowałam również, czy znaczny stopień agregacji ramet należących do tego samego osobnika genetycznego *B. humilis* utrudnia kojarzenia z innymi osobnikami tego gatunku.

Analiza markerów mikrosatelitarnych (ang. *simple sequence repeat*; SSR) przeprowadzona w populacji znajdującej się na terenie Bagna Wizna, jednego z największych zanikających torfowisk w Polsce, wykazała, że brzoza niska rozmnaża się tam wyłącznie klonalnie (Rozdział I). W całej populacji stwierdziłam tylko trzy genetycznie różne osobniki, co wskazuje na całkowity brak skutecznego rozmnażania płciowego. Analiza efektywności rozrodu i zróżnicowania genetycznego w loci AFLP (ang. *amplified fragment length polymorphism*), przeprowadzona w ośmiu marginalnych i trzech sub-centralnych populacjach

zlokalizowanych w Polsce i Białorusi, wykazała brak korelacji między zmiennością genetyczną a parametrami rozmnażania płciowego, takimi jak liczba kwiatów, masa nasion, zdolność nasion do kiełkowania (Rozdział II). Wydajność kiełkowania była jednak wyższa w populacjach sub-centralnych niż w marginalnych, a także na stanowiskach z wyższym poziomem wód gruntowych w porównaniu do siedlisk suchych. Oznacza to, że niesprzyjające warunki środowiska mogą w znacznym stopniu przyczynić się do spadku poziomu zmienności genetycznej brzozy niskiej, w szczególności w populacjach peryferyjnych. Markery AFLP posłużyły również do analizy częstości hybrydyzacji między brzozą niską a blisko spokrewnionymi z nią gatunkami brzóz drzewiastych: brzozą brodawkowatą *B. pendula* i brzozą omszoną *B. pubescens* (Rozdział III). W populacjach *B. humilis* stwierdziłam tylko trzy potencjalne hybrydy, jednak wszystkie pochodziły ze stanowisk suchych. Wynik ten sugeruje, że przy niskim poziomie wód gruntowych brzozy drzewiaste mogą zarastać brzozę niską, co skutkuje dominacją pyłku gatunków pospolitych i w konsekwencji zwiększonym prawdopodobieństwem zachodzenia kojarzeń międzygatunkowych. Analiza struktury klonalnej brzozy niskiej została przeprowadzona w sześciu populacjach, poprzez genotypowanie w sumie 522 ramet w siedmiu loci SSR (Rozdział IV). Badania wykazały, że ramety należące do tego samego osobnika klonalnego są zwykle zlokalizowane blisko siebie. Pomimo tego, znaczny poziom zróżnicowania klonalnego i genetycznego sugeruje, że kępowy typ wzrostu nie stanowi znaczącej przeszkody do zachodzenia zapyleń krzyżowych.

Przeprowadzone badania potwierdziły, że zmienność genetyczna marginalnych populacji *B. humilis* jest znacząca. Głównym czynnikiem odpowiedzialnym za zachowanie obecnych populacji są korzystne warunki środowiska, tj. wysoki poziom wód gruntowych, który uniemożliwia wkraczanie gatunków konkurencyjnych na tereny porośnięte przez brzozę niską.

Introduction

Marginal populations have long attracted the attention of biologists. At the edge of a species' geographical range, populations are expected to also be ecologically marginal (Lesica and Allendorf 1995). It was postulated that in ecologically deteriorated habitat conditions at the margins of species distributions, the population sizes and densities are decreased compared to those at the central localities. This pattern of intraspecific population structure was described as the 'abundant centre' hypothesis (Whittaker 1956, Eckert et al. 2008, Guo 2014). Harsh environmental conditions and low numbers of suitable mates in marginal populations can cause a decline in sexual reproduction, which in consequence leads to genetic erosion (Eckert et al. 2008). According to this point of view, genetically depleted populations inhabiting range edges are prone to extinction. However, phylogeographical studies revealed that patterns of genetic diversity within and between populations were mainly a consequence of changes in species' geographical distributions caused by recurring cyclical oscillation of the climate during the Quaternary (Hewitt 2004, Hampe and Petit 2005). Most European species survived the Last Glacial Maximum (LGM) in southern refugia located in Mediterranean regions (Jolly et al. 1998, Hewitt 2004), while cold-tolerant species survived at northern latitudes (Stewart and Lister 2001, Provan and Bennett 2008). During prolonged isolation in glacial refugia, populations accumulated many different mutations and, consequently, became genetically differentiated (Hampe and Petit 2005, Daneck et al. 2011). After climate warming, the species extended their ranges beyond LGM refugia. The postglacial recolonization process has caused genetic variation to decrease with increasing distance from the refugium due to founder effects (Hewitt 2000, Hampe and Petit 2005). However, as a result of the simultaneous expansion from separate refugia, migration waves could merge and mix together, forming secondary contact zones (Hewitt 2004). The contact zone of two phylogenetic lineages usually harbours even greater genetic variability than that observed in refugial populations (Petit et al. 2003, Havrdová et al. 2015). Nevertheless, the central-marginal model was adopted to describe distributions of genetic diversity throughout species' ranges (Eckert et al. 2008, Pironon et al. 2016).

Meta-analysis of central and peripheral populations belonging to 42 plant species revealed that marginality of the plants had a complex nature and did not follow one general

pattern (Abeli et al. 2014). For example, the genetic diversity of cedar glade *Leavenworthia stylosa* A. Gray populations generally supported the 'abundant centre' hypothesis, while the demographic variability was not consistent with its predictions (Dixon et al. 2013). This shows that often unclear interactions between local factors can be very important in shaping patterns of species genetic diversity formed after Holocene warming (Paun et al. 2008, Aikens and Roach 2014). The distribution of genetic variation of contemporary plant populations results from evolutionary forces (natural selection, genetic drift, mutations, and gene flow; Paun et al. 2008, Gitzendanner et al. 2012, Zellmer et al. 2012), habitat heterogeneity (Temunović et al. 2012, Gong and Gong 2016), human activities (Bartlewicz et al. 2015, Johnson et al. 2018) and the life-history traits of species (mating system, clonal propagation, and seed dispersal; Ahmed et al. 2009, Westergaard et al. 2011, Ozawa et al. 2013). Among these factors, different aspects of plant biology, i.e., sexual vs. asexual reproduction, seed and pollen dispersal, lifespan and hybridisation between closely related species, are considered to have the greatest effect on the genetic diversity and demographic performance of marginal populations (Abeli et al. 2014).

Effective generative reproduction can increase the level of genetic diversity through frequent recombination events, which create new gene combinations. However, depending on the species, peripheral populations can display very diverse sexual reproduction potentials. Abeli et al. (2014) showed that flower, fruit and seed production as well as the germination rate was similar across the species range in 40-60% of studies. For example, Eurasian steppe grass, *Stipa capillata* L., showed the same performance in terms of flower production and seed production, mass, size and viability in central and peripheral populations (Wagner et al. 2011). The germination rate, seedling mass and seed yield of the rare perennial plant *Lychnis viscaria* L. also did not differ significantly across the species' range (Lammi et al. 1999). In turn, in deerberry (*Vaccinium stamineum* L.) populations, seed mass even increased towards range limits, indicating local adaptation (Yakimowski and Eckert 2006). However, some peripheral populations can still exhibit lowered sexual reproductive performance due to less favourable environmental conditions. For example, a reduction in seed production was found in marginal populations of waterwillow (*Decodon verticillatus* (L.) Elliott) (Dorken and Eckert 2001) and three *Cirsium* species (Jump and Woodward 2003). A lower seed germination ability was noted in three highly isolated Polish stands of dwarf birch (*Betula nana* L.) than in centrally located Finish populations (Jadwiszczak et al. 2017). Wesche et al.

(2005) even found a lack of sexual reproduction at the edge of the distributional range of the clonal shrub *Juniperus sabina* L. With limited generative reproduction, extensive clonal propagation has been observed in marginal populations of numerous plants (e.g., Eckert and Barrett 1993, Jump and Woodward 2003, Beatty et al. 2008).

Klimeš et al. (1997) determined that 66.5% of central European plant species displayed some form of clonal growth. When sexual reproduction occurs simultaneously with vegetative propagation, the two reproductive modes can interfere with each other. Thus, the rate of sexual versus clonal reproduction in plant populations has a significant impact on their demography and genetic resources. Clonal architecture can significantly affect mating efficiency (Lovett-Doust 1981, Barrett 2015). Large clones with highly aggregated ramets exhibit an increased probability of self-pollination by geitonogamy (Harder and Barrett 1996, Eckert 2000, Barrett 2015). For example, the selfing rate in *Vaccinium myrtillus* L. was significantly higher for plants growing in plots with a low number of distinct genets than in plots shared by numerous intermingled clones (Albert et al. 2008). Therefore, limited gene exchange between highly compacted genets is considered a potential factor inducing significant spatial genetic structure (SGS), as more intense SGS was found in populations of clonal species than in those of non-clonal species (Dering et al. 2015).

Individuals in highly clonal self-incompatible plant populations can display low levels of genetic diversity and share the same incompatibility alleles (Honnay and Jacquemyn 2008, Gitzendanner et al. 2012). This can preclude finding proper mates of the same species, thereby increasing the probability of crossing with closely related congeners. Hybridisation can have a significant impact on the genetic structure of plant populations, as it was estimated that approximately 25% of plant species undergo this process (Mallet 2005). Hybridisation is the crossbreeding of individuals from genetically distinct populations caused by a lack of definite reproduction barriers between species coexisting in sympatric populations (Mallet 2007, Abbott et al. 2013). When reproductive isolation between hybrid and parental species is not complete, it can lead to introgression, which allows the transfer of neutral or adaptive genetic traits between species and an increase in genetic variation in populations that come into contact (Rieseberg and Carney 1998). For example, hybridisation between endemic *Cyclamen balearicum* Willk. and widespread *Cyclamen repandum* Sm. was suggested to explain the higher genetic and floral variability in locations with both species than in single-species sites (Thompson et al. 2010). Although some degree of gene flow may be

evolutionarily favourable, hybridisation and introgression may also cause disruption of gene complexes responsible for local adaptation and the disappearance of pure populations of certain species. Consequently, they may contribute to the decline of rare plant species at range margins, which is particularly a threat for endangered species coming into contact with more abundant ones (Rhymer and Simberloff 1996, Largiader 2007).

One of the species with a range that reaches its geographical boundary in the territory of Poland is the shrub birch *Betula humilis* Schrk. The European continuous range of *B. humilis* extends from western Siberia and north-western Mongolia to north- and south-eastern parts of Poland (Ashburner and McAllister 2016). The presence of remnant populations in the Alps, the Carpathians and northern Germany implies that shrub birch was more widespread in the past (Hultén and Fries 1986, Załuski et al. 2014). *Betula humilis* is recognized as a glacial relict in central and western Europe and is listed as an endangered (EN) species in red plant books in Belarus, the Czech Republic, Germany, Poland, Ukraine, and the Kaliningrad region of Russia (Calko 2014, Załuski et al. 2014). Shrub birch is a much-branched, monoecious, wind-pollinated and wind-dispersed species that also reproduces vegetatively. The number of *B. humilis* populations in Poland decreased significantly during the twentieth century, from approximately 350 to only 70 (Załuski et al. 2014). Although the species can form abundant populations in dry and wet areas (Jabłońska 2006, 2012), its expansion depends strongly on solar radiation. It is a poor competitor, and it declines in dry habitats, where brushwood and forest plants are at a selective advantage (Jabłońska 2006, 2012). Many Polish populations of shrub birch are isolated from one another; thus, seed and pollen dispersal between populations seems impossible, especially since shrub birch forms low bushes that are usually surrounded by tall forest trees. Among vascular plants, the *Betula* genus was recognised as one of the taxa most involved in hybridisation (Whitney et al. 2010, Barrington 2011); hence, crossbreeding between *B. humilis* and the widespread tree birches *Betula pendula* Roth and *Betula pubescens* Ehrh. seems to be another threat for small and overgrown shrub birch stands. However, despite these limitations, a high level of genetic diversity, comparable to that observed in the sub-central localities in Belarus, was recorded in the present-day populations of *B. humilis* located in north-eastern Poland (Jadwiszczak et al. 2011a, 2012). The primary reason for this considerable genetic variation is likely the heritage of the admixture zone. Most likely, the territory of central Europe was colonised by migration waves of birches, deriving from at least two distinct glacial refugia that came into contact and formed an

admixture zone in Poland (Palmé et al. 2003, Jadwiszczak 2012, Jadwiszczak et al. 2012, 2015a, b). On the other hand, in the smallest and most isolated populations, low genetic variation resulting from genetic drift and reduced gene flow were noted (Jadwiszczak et al. 2011a, b).

Considering these issues, the primary aim of my PhD thesis was to define the factors that currently shape the genetic diversity of *B. humilis* populations at the south-western margin of its range. The high variability of chloroplast DNA (cpDNA) and microsatellite markers detected in some shrub birch localities in Poland (Jadwiszczak et al. 2011a, 2012) strongly suggested that effective sexual reproduction occurred in the populations. Thus, I hypothesized that **sexual reproduction efficiency would not differ between peripheral and sub-central *B. humilis* localities** (Hypothesis 1, Chapter II). Moreover, I tested if **the reproductive performance of the shrub birch was dependent on environmental conditions** (Hypothesis 2, Chapter II). I also assumed that **interspecific gene exchange would increase the genetic diversity of marginal *B. humilis* populations** (Hypothesis 3, Chapter III). Another aim of the studies was to verify if **aggregated clonal growth could decrease crossing between genetically diverse individuals** (Hypothesis 4, Chapters I and IV).

Chapter I

Disappearing population of *Betula humilis* Schrk. on the Maliszewskie Lake, NE Poland

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Disappearing population of *Betula humilis* Schrk. on the Maliszewskie Lake, NE Poland

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Abstract: *Betula humilis* Schrk. is an endangered glacial relict inhabiting wet meadows, natural and drained fens. One of its declining populations is located on the Maliszewskie Lake (the Wizna swamp, north-eastern Poland). The goal of the present study was to estimate the number of *B. humilis* individuals in this locality. In the Maliszewskie Lake population, 59 ramets, grouped into three clusters, were found. Twelve nuclear microsatellite loci were chosen to genotype 52 ramets. The analysis revealed that all the shoots within the single cluster had the same genotypes at the loci considered. This means that each cluster constituted one genetically distinct individual; thus, there were only three individuals of *B. humilis* in the studied population. The maintenance of the *B. humilis* population in the Maliszewskie Lake area requires urgent active protection involving removal of the shading vegetation. In fact, the entire Maliszewskie Lake is worthy of protection because of its hitherto unexplained origin and the occurrence of many endangered bird species.

Key words: *Betula humilis*, conservation genetics, genet, microsatellite, ramet, wetland conservation

1. Introduction

In land use classification, wetlands are known as wastelands in terms of both agriculture and forestry. However, biologists postulate that they are significant biodiversity units because the combination of aquatic and terrestrial conditions allows the existence of a variety of animal and plant organisms, including many endangered species (Bacon 1997; Fujita *et al*. 2014). Wetlands also play a very important role in water retention, limitation of soil erosion, lowering the risk of flood and in water quality improvement by serving as filters. In addition, wetlands act as resting and feeding places for some birds during their spring and autumn migrations. Unfortunately, the area of the world's swamps has declined by half during recent centuries (Keddy 2000). In Poland, the loss of mires has reached more than 80% (Wolejko *et al*. 2005), which is mainly a consequence of drainage. The overgrowth of drained fens and meadows by reeds, shrubs and trees reduces their areas and initiates the decline of mire habitats. These processes could have a disastrous impact on wetland species, ranging from reductions in the numbers of individuals to a complete disappearance of populations.

Wizna swamp is one of the biggest declining fens in Poland. Until the First World War, the central part of the mire, drained by poor systems of ditches, was used to a small extent by farmers who mowed the grass for cattle and horses (Kołos & Próchnicki 2004). During this time, some parts of the swamp were particularly valuable in terms of natural diversity, as they were populated by numerous specimens representing rare plant species in Poland, including *Herminium monorchis*, *Pedicularis sceptrum-carolinum*, *Schoenus ferrugineus*, *Swertia perennis* and two sedge species, *Carex chordorriza* and *C. limosa* (see Kołos & Próchnicki 2004). The abandonment of mowing after the Second World War caused the overgrowth of the Wizna swamp by bushes. However, drainage conducted in the whole fen from 1962 to 1971 changed this region completely. Previous communities of bushes, dominated by *Betula pubescens*, *Frangula alnus*, *Populus tremula*, *Salix cinerea*, *Viburnum opulus* and two glacial relicts, *Betula humilis* and *Salix lapponum,* were extirpated and replaced by high fodder productivity grasslands (Kołos & Próchnicki 2004). Almost 6,000 hectares of drained mire were included into the State Agricultural Farm "Wizna".

Although the adverse effects of drainage are visible in all parts of the Wizna mire, there are three

parts that are relatively undisturbed, namely: "Biel" and "Grzędy" ranges as well as the vicinity of the Maliszewskie Lake (Kołos & Próchnicki 2004). Some authors have suggested that the Maliszewskie Lake could be one of four old glacial water reservoirs in the Północnopodlaska Plain (north-eastern Poland), and the only one in the Narew river valley (Banaszuk 2004; Kołos & Tarasewicz 2005). It is surrounded by topogenic-soligenic mire, where *B. humilis* can still be found. *B. humilis* has been classified in the EN (endangered) category of the International Union for Conservation of Nature (IUCN) in central and western Europe (Załuski *et al*. 2014). The disappearance of its populations is mainly a consequence of the lowering of groundwater levels and a decline in the use of wet meadows. An inventory carried out at the end of the twentieth century revealed that the number of *B. humilis* stands in Poland diminished to approximately 20% of the former number (Załuski *et al*. 2014). The *B. humilis* population located on the Maliszewskie Lake also seems to be threatened because the lake is becoming smaller and shallower, which are both effects of the drainage conducted in the 1960s. Environmental monitoring by the General Inspection of Nature Protection showed that the ecological status of the lake was unsatisfactory (U1 category; Wilk-Woźniak *et al*. 2012). Specifically, the reduction of the lake surface and shallowing caused the overgrowth of trees and scrub vegetation. Consequently, the growth of the light-demanding *B. humilis* is disadvantaged in the shaded stands, and its population has declined in such places (Jabłońska 2012). The goal of this paper is to estimate the number of *B. humilis* individuals in the Maliszewskie Lake population and to propose suitable conservation practices.

2. Material and methods

The studied *B. humilis* population is located on the western side of the Maliszewskie Lake (N 53º10´07.8´´, E 22º30´45.5´´), ca. 44 km west of the city of Białystok (Fig. 1). The marginal zone of the lake is dominated by *Thelypteridi-Phragmitetum* and *Phragmitetum australis* (Kołos & Próchnicki 2004). Bulrush vegetation directly surrounding the lake is separated from the adjacent fields and meadows by a narrow belt of forest with *Betula pubescens* and *Salix cinerea*. Three clusters of *B. humilis* shoots were found in this forest. The clusters were designated as follows: A, B and C. In total, 59 ramets were counted in the three clusters, with nine in cluster A, 23 in B and 27 in C. Some ramets were very young and small. Thus, one leaf was taken from every ramet having more than three leaves. Altogether, 52 ramets were sampled, of which eight were in cluster A, 21 – in B and 23 – in C (Table 1). The samples were collected with the permission of the Regional Director of Environmental Protection in Białystok (WPN. 6400.45.2013.AP).

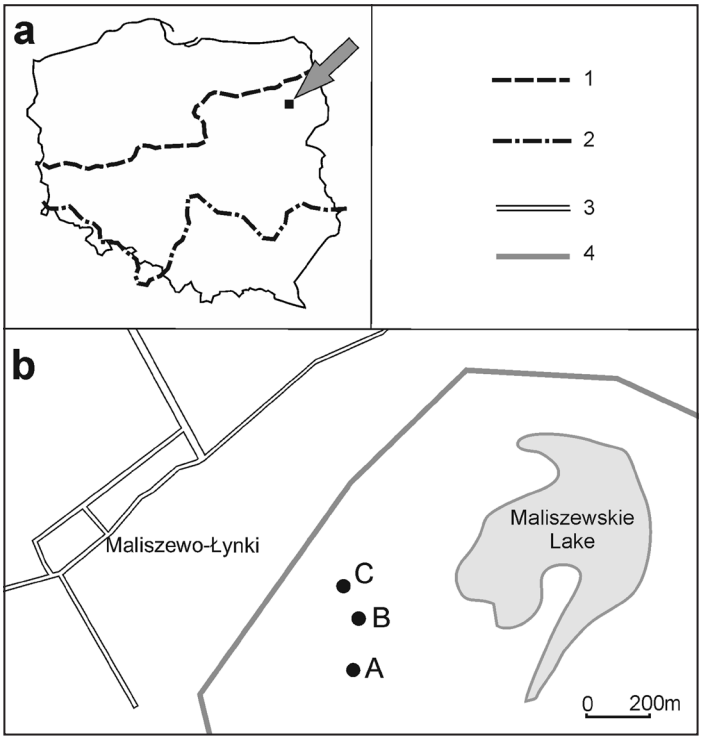

Fig. 1. Location of *Betula humilis* population in NE Poland (a), on the Maliszewskie Lake (b)
Explanations: A, B and C – location of genetic clusters of *B. humilis* on the Maliszewskie Lake; 1 – limit of the ice-sheet in the Vistulian Glaciation, 2 – limit of the ice-sheet in the Odranian Glaciation, 3 – road, 4 – ditch

The leaf material was transferred to the laboratory in a car refrigerator and stored at – 80ºC until analysis. Before DNA extraction, leaves were dried at room temperature for one day. Next, they were homogenised with the TissueLyser mill (Qiagen) using steel balls. Total genomic DNA was extracted using an AX Plant Kit (A&A Biotechnology), according to the manufacturer's procedure. To determine the genotype of each ramet, 12 nuclear microsatellite primer pairs, originally designed for *B. pendula* (L1.10, L2.7, L13.1, L5.4, L4.4, 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), were chosen. The combinations of primers into four multiplex PCRs, the proportions of the PCR reaction components and the PCR profile for each multiplex were as previously described by Jadwiszczak *et al*. (2011a). The separation of fluorescently labelled amplified fragments was conducted on an ABI PRISM 3130 sequencer (Applied Biosystems) and scored using GeneMapper 4.0 (Applied Biosystems) analysis software.

3. Results and discussion

Successful amplification was obtained for all nuclear microsatellite loci of the *B. humilis* ramets studied, except for the L4.4 locus in the cluster B (Table 1). The lack of amplification in B ramets at the L4.4 locus likely resulted from mutations occurring at primer sites, leading to the appearance of null alleles (van Oosterhout *et al*. 2004). This result strongly suggests that the B ramets had the same homozygous genotype at this locus. Further analysis revealed that all shoots belonging to the same cluster had identical alleles at all the microsatellite loci considered. This means that each cluster constituted one genetically distinct individual (genet); hence, there were only three specimens of *B. humilis* in the Maliszewskie Lake population. A similar result was previously obtained in the four populations of endangered *Haloragodendron lucasii* (northern Sydney, New South Wales, Australia), where 53 ramets were sampled and only six multilocus genotypes were observed at allozyme and RAPD loci (Sydes & Peakall 1998).

All individuals in the Maliszewskie Lake population were homozygotes at L13.1 and L2.3 loci. In general, these loci showed a very low level of polymorphism in *B. humilis*, as the previous analysis of 327 specimens from 18 populations from Poland and Belarus revealed four alleles at the L13.1 locus and three at L2.3 (Jadwiszczak *et al*. 2011a). Loci L2.7, L5.4, L5.1, Bo.F394 and L022 were heterozygous in all the analysed genets, and the remaining loci were heterozygous or homozygous, depending on the individual studied. Allele sizes of particular microsatellites detected in the Maliszewskie Lake population were in the size ranges described previously in other *B. humilis* locations (Jadwiszczak *et al*. 2011a, 2011b). We did not find any unique or private allele in the Maliszewskie Lake stand; however, this result was not surprising. The population of *B. humilis* in the Wizna mire underwent a severe reduction in numbers during drainage in 1960s. Low frequency alleles are lost rapidly during bottlenecks because the elimination of any specimen having unique alleles in their genotype results in the disappearance of such alleles (Nei *et al*. 1975; Luikart & Cornuet 2008). Moreover, the effective population size of the bottlenecked populations is significantly reduced, which consequently causes a further reduction in the allele number (Cornuet & Luikart 1996).

Unfortunately, genetic erosion, which occurs characteristically in small populations, can dramatically influence their resistance to diseases and parasites, as well as their ability to cope with environmental changes (Ellstrand & Elam 1993; Lacy 1997). In general, *B. humilis* shows a wide spectrum of ecological tolerance. Jabłońska (2012) distinguished the following seven types of habitats populated by the species: *Sphagnum* moss-small sedge poor fens with a high contribution of bog species from the *Oxycocco-Sphagnetea* class,

Table 1. Genotypes at the nuclear microsatellite loci of *Betula humilis* ramets collected in the three clusters on the Maliszewskie Lake

Cluster	No of ramets	Microsatellite loci											
		L 1.10	L 2.7	L 13.1	L 5.4	L 4.4	L 5.1	Bo.G 182	Bo.F 394	L 3.1	L 2.3	L 021	L 022
A	8	177	175	080	247	279	286	129	140	215	198	190	171
		177	179	080	253	279	300	129	150	217	198	192	195
B	21	187	175	080	235	na	298	127	134	217	198	194	181
		191	179	080	247	na	326	133	148	217	198	200	197
C	23	175	173	080	241	263	288	129	148	217	198	200	179
		187	183	080	257	271	300	133	170	217	198	200	203

Explanation: na – no amplification

brown moss-small sedge subneutral fens with the highest number of mesotrophic subneutral fen species from the Scheuchzerio-Caricetea nigrae class, brown moss-small sedge alkaline fens with species from the Caricion davallianae alliance, strongly degraded fens dominated by *Urtica dioica* and *Galium aparine,* spring mires with water rich in Mg^{2+} and Ca^{2+} ions and alkaline fen meadows. It was found that the maintenance of *B. humilis* in its habitats depended on calcium concentration and water level. Both high Ca^{2+} concentrations and high water levels prevented the spread of other plants and enabled the growth of light-demanding *B. humilis* (Jabłońska 2006). In the Maliszewskie Lake population, the concentration of calcium ions is rather average, compared to other *B. humilis* localities (Jabłońska 2009; Jadwiszczak *et al*. 2015), which could have weakened the competitive ability of this species. However, the water table in the Maliszewskie Lake seems to be advantageous for the species, as it is around the peat surface (Jabłońska 2009, 2012). In addition to the relatively high water table, *B. humilis* in the Maliszewskie Lake clearly suffers due to shading by other shrub and tree species. It is likely that the dominance of shading vegetation results from year to year variations in the water level in this locality, which depends on rainfall. In dry years, brushwood and forest species might spread increasingly and displace *B. humilis*. In the undisturbed mires, e.g., the Rospuda mire in north-eastern Poland, stable hydrologic conditions allow the existence of a stable and long-lasting *B. humilis* population (Jabłońska *et al*. 2011).

Populations of *B. humilis* located in north-eastern Poland are located mainly in national parks, landscape parks or reserves. However, active conservation practices are not used in these locations (Matowicka & Jabłońska 2008). It is obvious that the maintenance of *B. humilis* on the Maliszewskie Lake requires urgent active protection, such as the removal of brushwood and forest plants, at least around the existing clusters of the species. This should be followed by water retention enhancement, which should stop the succession of other species. In the present habitat conditions, the transplantation of *B. humilis* individuals from the adjacent populations is not recommended because there is little chance for their acclimation.

The Maliszewskie Lake is a unique place not only because of the presence of the endangered glacial relict *B. humilis*. In addition, its unexplained origin and huge richness of birds make it extremely valuable for conservation. The results of palynological investigations suggest that the Maliszewskie Lake arose as a result of melting of ground ice blocks (Stasiak 1979; Żurek *et al*. 2002; Banaszuk 2004). However, that part of Poland was not covered by ice sheets during the last glaciation (Fig. 1). Could the lake be dated back to the third of the Middle Polish Glaciations, the Odranian Glaciation (210-130 ka BP)? This hypothesis seems to be confirmed by a relatively small area of the lake, its shallowness (the maximum depth is 80 cm) and its very thick layer of sediments (22.5 m; Stasiak 1979). However, the beginning of biogenic layer formation was previously dated to the Alleröd interstadial of the Late Vistulian (see Żurek *et al*. 2002).

The Maliszewskie Lake is a paradise for ornithologists. Among others, such endangered bird species as: *Philomachus pugnax* and *Acrocephalus paludicola* can be observed here (Zakrzewska 2010). As such, the conservation of the Maliszewskie Lake habitat and increasing publicity could help to relieve and protect the adjacent Biebrza National Park, which is particularly important during the spring migration of birds, when large numbers of tourists and bird-watchers visit the park (Zakrzewska 2010).

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Chapter II

Sexual reproduction efficiency and genetic diversity of endangered *Betula humilis* Schrk. populations from edge and sub-central parts of its range

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Sexual reproduction efficiency and genetic diversity of endangered *Betula humilis* Schrk. populations from edge and sub-central parts of its range

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Abstract Outcrossed mating systems play a very important role in the persistence of endangered, self-incompatible plants such as shrub birch, *Betula humilis* Schrk. The goal of this study was to estimate, for the first time, the effectiveness of sexual reproduction in threatened edge and sub-central populations of shrub birch. The amplified fragment length polymorphism (AFLP) method revealed that all of the individuals at each locality had different genotypes. The matrix incompatibility count (MIC) suggested that the effectiveness of recombination was similar among all the populations of shrub birch under study. However, taking into account the greater germination ability of seeds in sub-central populations, we conclude that sexual reproduction in those populations can be more efficient. The germination capacity of seeds depends on their mass, which was significantly lower in dry or more shaded sites compared to wet or more exposed sites. Non-significant results of multiple regression models suggest that chemical parameters of the habitat (pH, EC, NH_4^+, PO_4^{3-}) had no influence on the reproductive output of *B. humilis*. The discrepancy between the still quite substantial genetic diversity and the poor sexual reproduction in shrub birch populations can be explained by the production of a few phosphorus-rich seeds, insufficient time for a decrease in genetic variation in the disappearing part of the range or hybridization with close congeners.

Keywords AFLP · *Betula humilis* · habitat parameters · matrix incompatibility count · recombination · sexual reproduction

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Introduction

In its updated Global Strategy for Plant Conservation, the Convention on Biological Diversity demanded protection for 60 % of the world's plant species (Joppa et al. 2013). It is widely accepted that developing the most effective conservation strategies for endangered species requires knowledge of genetic resources and the mechanisms responsible for their distribution within species ranges (Rao and Hodgkin 2002). The primary distributions of species genetic diversity are the outcome of range contractions during the stadials of the Pleistocene glaciations and of expansions during the interstadials (Hewitt 1999; Petit et al. 2003). However, the patterns of genetic structuring within and between populations established after the glacial retreat have since been modified by the life history traits of species (e.g. mating system, clonal reproduction and seed dispersal – Glémin

et al. 2006; Alsos et al. 2012; De Witte et al. 2012), the ongoing evolutionary processes that shape current populations (e.g. mutation, selection, genetic drift and gene flow – Mitton and Duran 2004; Jump et al. 2006; Jadwiszczak et al. 2012b) as well as fragmentation of habitats resulting mainly from anthropopression (Jump and Peñuelas 2006).

Among the biological characteristics of plant species, mating systems play an especially significant role in the persistence of populations because they shape genetic structure via the transmission of genes across generations, determine the rate of genetic diversity loss and influence the evolution of traits (Hamrick and Godt 1996; Neel et al. 2001; Glémin et al. 2006). Clonally reproducing self-incompatible plants seem to be particularly threatened due to the low number of genetically distinct individuals (genets) in a population, which diminishes the chances for conspecific pollination with compatible mates. In plant populations with incomplete reproductive barriers, the second danger is an increased probability of interspecific breeding with common congeners that could result in either the production of inviable seeds or the displacement of rare species by overdominant hybrid progeny (Nagamitsu et al. 2006). All of the above situations could drive small populations to extinction.

The purpose of this study was to determine the factors influencing the reproductive success and genetic diversity of populations of shrub birch, *Betula humilis*, located at the edge (Poland) and in sub-central (Belarus) parts of its range. *Betula humilis* is a much-branched shrub with dark brown bark covered with numerous white resin glands. Like other birches, this plant is light-demanding, and its growth depends on the intensity of solar radiation. The bushes are usually not higher than 1–2 m, but they can reach a height of 3–4 m in heavily shaded positions. The species inhabits seven main types of habitats: *Sphagnum* moss-small sedge poor fens with a high contribution of bog species from the class *Oxycocco-Sphagnetea*, brown moss-small sedge subneutral fens with the highest number of rich-fen species from the class *Scheuchzerio-Caricetea nigrae*, brown moss-small sedge alkaline fens with species from the *Caricion davalianae* alliance, strongly degraded fens dominated by *Urtica dioica* and *Galium aparine*, spring mires with water rich in Mg^{2+} and Ca^{2+} ions, and alkaline fen meadows (Jabłońska 2012). Because drainage benefits the plant in some way, *B. humilis* can also be abundant in moderately or even intensively drained variants of natural habitats. The chemical properties of groundwater sampled in *B. humilis* populations are differentiated: pH 3.7–8, 50–1,200 $\mu Sm \cdot cm^{-1}$ EC, 5–130 $mg \cdot l^{-1}$ Ca^{2+}, 0.5–20 $mg \cdot l^{-1}$ Mg^{2+}, 0–10 $mg \cdot l^{-1}$ $N\text{-}NH_4^+$ and 0–0.65 $mg \cdot l^{-1}$ $P\text{-}PO_4^{3-}$ (Jabłońska 2012).

Shrub birch is under strict protection in Poland, Germany, Ukraine and the Kaliningrad Oblast of north-western Russia. In Poland, the number of populations has decreased approximately fourfold during the twentieth century (Załuski et al. 2014). In 2014, the species was also included on the list of rare and endangered species in Belarus (Calko 2014). Despite the contraction of the range of shrub birch, previous nuclear microsatellite analyses have revealed a still reasonably high level of intra-population genetic diversity, low to average inter-population differentiation and non-significant genetic differences between edge Polish and sub-central Belarusian populations (Jadwiszczak et al. 2011a,b). The distributions of chloroplast DNA and nuclear *ADH* (alcohol dehydrogenase) gene haplotypes strongly imply that the study territory was populated through waves of migrations from distinct glacial refugia, so the substantial genetic diversity could have resulted from the formation of an admixture zone (Jadwiszczak et al. 2012a, 2015a,b). The shapes of the cpDNA and *ADH* minimum spanning trees indicate a rapid increase in population size during recent expansions of the species' range and low or modest gene flow between contemporary populations (Jadwiszczak et al. 2012a, 2015b).

Like many rare species, *B. humilis* is a poor competitor, so it is more abundant in sites where other plants cannot develop, i.e. places with high concentrations of calcium ions and high water levels (Jabłońska 2006, 2012). However, little is known about the current evolutionary forces acting on shrub birch populations. The relatively low genetic diversity and significant differentiation among some of the smallest and most isolated populations from northern Poland suggest that they are subjected to strong genetic drift and limited gene flow (Jadwiszczak et al. 2011a). Additionally, selection may act against heterozygous genotypes in calcium-rich habitats, as a significant negative correlation was found between the observed heterozygosity of microsatellite loci and Ca^{2+} concentrations (Jadwiszczak et al. 2015b). To the best of our knowledge, there is no information in the literature on the effectiveness of sexual reproduction of *B. humilis*. Shrub birch is a monoecious, wind-

pollinated and wind-dispersed species, and based on annual seed production (Załuski et al. 2014) and the substantial variation at microsatellite loci (Jadwiszczak et al. 2011a,b), it has been assumed that sexual reproduction is prevalent in populations located at the south-western edge of the range. However, analysis of nuclear microsatellite genotypes has indicated that the species spreads only clonally in unfavourable habitats (Chrzanowska and Jadwiszczak 2015). Our aims were to: (1) study if flower and seed productions vary significantly among *B. humilis* populations in edge and sub-central parts of its range, (2) determine the relative importance of environmental conditions for the sexual reproduction of the species, and (3) investigate the relationships between sexual reproduction and genetic variation parameters. Genetic diversity in the populations studied was assessed using the amplified fragment length polymorphism (AFLP) method. Of eleven populations, nuclear microsatellites had previously been investigated at ten sites (Jadwiszczak et al. 2011a,b), but the AFLP method, which can discover hundreds of DNA loci throughout the whole genome, is a more effective tool than microsatellites to test for clonal identity between individuals and thus to draw conclusions about sexual and asexual modes of reproduction (Mueller and Wolfenbarger 1999; Kameyama and Ohara 2006; Majeský et al. 2012).

Material and methods

Sample collection and chemical analyses of groundwater

The study was conducted in eight edge Polish and three sub-central Belarusian populations of *B. humilis* (Table 1, Fig. 1). Each sampling locality was visited twice in 2012: in the spring to sample fresh leaves for DNA isolation and to count the flowers, and in the autumn to collect seeds (Table 2). In total, 254 individuals were analysed to determine the presence of female and male flowers; of those, 220 specimens were sampled for the genetic analyses, and 168 shrubs (all with seed coins) were used for the germination experiment. To minimize the probability of collecting vegetative ramets, the minimum distance between adjacent samples was 20 m. Sample collection in Poland was approved by the Polish Ministry of the Environment (DOPpn-4102-873/41255/11/RS) as well as by the Regional Directors of Environmental Protection in Białystok (WPN.6202.15.27.2011.MW), Lublin (WPN.6205.60.2011.MO), Bydgoszcz (WPN.6402.1.16.2011.JC) and Gdańsk (RDOŚ-Gd-PNII.6402.1.80.2011.KD). Shrub birch was not classified as endangered in Belarus at the time of the study. The leaf material collected for DNA isolation was dried in silica gel and stored at room temperature. Seeds were collected in paper bags and also kept at room temperature.

For each population, six to 10 habitat plots were established during the spring and autumn to sample groundwater for chemical analyses, including pH, electrical conductivity (EC; $\mu Sm \cdot cm^{-1}$), the concentrations of NH_4^+ ($mg \cdot l^{-1}$) and PO_4^{3-} ($mg \cdot l^{-1}$) ions, and groundwater level (Table 2). EC and pH were measured directly in the field using an EC-60 electrode and an EPS-1 electrode, respectively, which were connected to a CPC-401 pH-meter. The concentrations of NH_4^+ and PO_4^{3-} were determined through colourimetric analysis in the laboratory using the PhosVer HACH reagent and the salicylate method, respectively. Based on Jabłońska's (2012) observations that *B. humilis* prefers water levels near the surface of the peat, the groundwater level in each sampling plot was assessed as either high (HWL; ≥ -10 cm) or low (LWL; ≤ -10 cm), and the degree of shading was arbitrarily estimated as no shade (no canopy cover), half shade (canopy cover of 50 %) or full shade (canopy cover of 100 %).

DNA laboratory analyses

After homogenization of the leaf material in a TissueLyser LT bead mill (Qiagen), DNA was extracted with the AX Plant Kit (A&A Biotechnology) according to the manufacturer's instructions, and the samples were then genotyped for AFLP markers. The AFLP procedure followed that of Vos et al. (1995) with some modifications suggested by Applied Biosystems (AFLP Plant Mapping Protocol). First, 33 primer pair combinations were tested on four individuals selected from the most distant populations, and the three primer combinations that resulted in the most polymorphic and repeatable fragments of homogenous intensity were chosen: *Eco*RI-ACC/*Mse*I-CAC, *Eco*RI-ACC/*Mse*I-CAT and *Eco*RI-AGC/*Mse*I-CAC. The NED-labelled products of the selected amplifications were separated using an ABI PRISM 3130 (Applied Biosystems) instrument with a GeneScan 500 LIZ Size Standard (Applied Biosystems). AFLP profiles were scored for the

Table 1 Names of sub-central (1–3) and edge (4–11) populations of *B. humilis* studied, their geographical locations, number of individuals sampled for genetic analyses, genetic diversity parameters (PLP – proportion of polymorphic fragments, *H* – Nei's gene diversity) and inbreeding coefficients (f_{AFLP}).

	Population	Code	Location		No. of inds.	PLP	$H \pm SE$	f_{AFLP}
			Latitude	Longitude				
1.	Belarus, Ozero Borovoe	BOR	52.83′ N	28.78′ E	21	33.3	0.096 ± 0.010	−0.024
2.	Belarus, Berezin'skij Zapovednik	BZ	54.63′ N	28.35′ E	20	32.9	0.107 ± 0.011	0.024
3.	Belarus, Sluck	SLU	53.29′ N	27.63′ E	21	32.9	0.103 ± 0.011	−0.028
4.	Poland, Jezioro Mętne	JM	53.79′ N	17.77′ E	20	31.1	0.101 ± 0.011	0.014
5.	Poland, Magdzie Bagno	MB	54.08′ N	23.16′ E	21	37.9	0.113 ± 0.010	−0.006
6.	Poland, Bagno Bubnów	BB	51.22′ N	23.16′ E	21	36.5	0.114 ± 0.011	0.000
7.	Poland, Torfowisko Sobowice	TS	51.07′ N	23.23′ E	13	30.1	0.120 ± 0.012	0.012
8.	Poland, Uroczysko Uściwierskie	UU	51.21′ N	23.03′ E	21	36.1	0.110 ± 0.011	0.020
9.	Poland, Łąki Ślesińskie	LS	53.13′ N	17.70′ E	21	35.6	0.111 ± 0.011	0.014
10.	Poland, Czerwone Bagno	CB	53.62′ N	22.82′ E	20	35.2	0.108 ± 0.011	0.003
11.	Poland, Szuszalewo	SUS	53.73′ N	23.36′ E	21	36.5	0.108 ± 0.010	0.019

presence or absence (1 or 0, respectively) of bands between 70 and 500 bp using GENEMAPPER 4.0 software (Applied Biosystems). To confirm the reproducibility of the AFLP fragments, two individuals from each population were replicated starting from the restriction/ligation reaction. The error rate was estimated as the percentage of different scores in comparison of 1/0 matrices obtained for the duplicated samples (Bonin et al. 2004).

Germination experiment

From each of the individuals used to study seed quality, 100 seeds were selected and collectively weighed (wet mass) with an accuracy of 0.0001 g. In total, 16,800 seeds were taken to the germination experiment. To break dormancy and increase the probability of germination, the seeds were kept at low temperatures from mid-November until the beginning of April: a total of

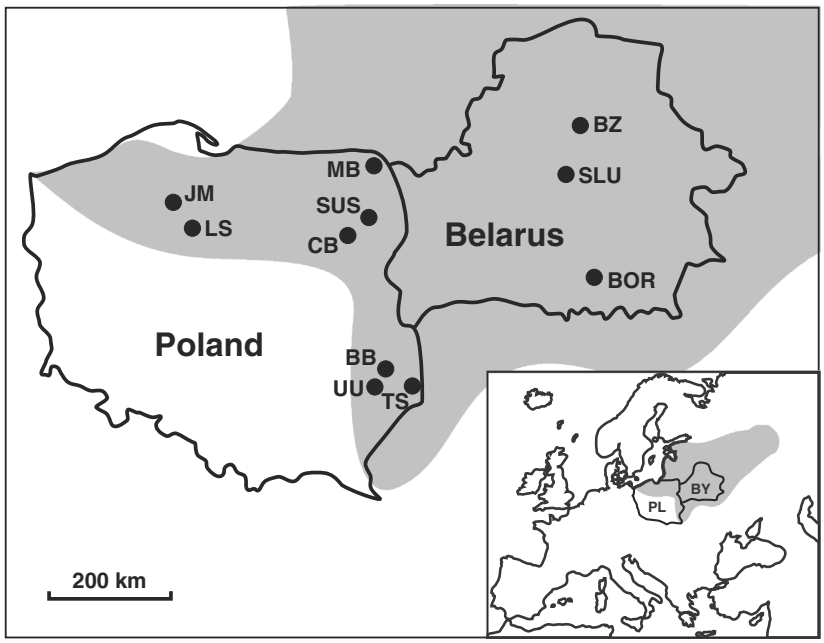

Fig. 1 Locations of edge Polish and sub-central Belarusian sampling sites of *B. humilis*. Population codes according to Table 1. The grey area indicates the European range of the species.

Table 2 Median values of the parameters of habitat quality and reproductive output in the *B. humilis* populations. Pop – population codes according to Table 1 (1–3 sub-central and 4–11 edge populations); Habitat quality parameters: EC – electrical conductivity; Shade: 0 – no shade (no canopy cover), 1 – half shade (canopy cover of 50 %), 2 – full shade (canopy cover of 100 %); Water level: HWL – high (≥ −10 cm), LWL – low (≤ −10 cm); Number of flowers per flowering individual: f – female flowers, m – male flowers; GS – number of germinated seeds per individual.

	Pop.	No. of habitat plots	Habitat quality parameters						Reproduction parameters							
									Flowering					Seed quality		
			pH	EC [µS cm^{-1}]	NH_4^+ [mg l^{-1}]	PO_4^{3-} [mg l^{-1}]	Shade	Water level	Total No. of inds	No. of inds with flowers	No. of flowers per flowering ind.			No. of inds	Weight of 100 seeds [g]	GS
											Total	f	m			
1.	BOR	10	4.45	53.5	1.04	0.117	1	HWL	21	9	1.5	2.0	0.0	9	0.0117	1.0
2.	BZ	10	6.65	261.0	1.02	0.107	0	HWL	25	25	13.5	14.0	0.0	25	0.0202	27.0
3.	SLU	10	6.95	519.0	1.28	0.129	0	LWL	25	24	20.0	16.5	0.0	23	0.0173	32.0
4.	JM	10	3.95	95.0	1.06	0.201	1	HWL	25	21	40.0	11.0	20.0	19	0.0165	11.0
5.	MB	10	5.95	135.5	0.82	0.159	1	HWL	25	24	18.0	11.0	6.0	23	0.0179	11.0
6.	BB	10	6.75	482.0	0.55	0.102	0	LWL	24	11	7.5	3.0	2.0	9	0.0177	0.0
7.	TS	6	7.00	948.0	0.41	0.124	1	LWL	13	11	14.0	7.0	6.0	8	0.0124	1.0
8.	UU	8	6.10	230.0	1.13	0.258	2	LWL	21	12	88.0	12.5	78.0	9	0.0112	1.0
9.	LS	10	6.90	10, 34.5	0.68	0.179	2	LWL	25	13	23.0	10.0	14.0	13	0.0152	1.0
10.	CB	10	6.00	216.0	1.12	0.177	2	LWL	25	14	5.0	4.5	0.0	14	0.0113	1.0
11.	SUS	10	6.60	229.5	0.84	0.086	1	HWL	25	19	5.0	4.0	1.0	16	0.0188	16.5

three months at 4°C and three weeks in February at −20°C. After vernalization, seeds were placed in closed Petri dishes on wet filter paper, and a germination test was conducted in a phytotron at the constant temperature of 20°C with a photoperiod of 10 hrs light/14 hrs dark (Holm 1994). Germinated seeds (those with visible radicles) were counted each day and removed, and the experiment was completed after 42 days, at which time no seed had germinated for five days (Holm 1994). During the test, distilled water was topped up regularly.

Statistical analyses

The AFLPdat R-script (Ehrich 2006) was run to check for the potential resampling of clones. For each population, allele frequencies were calculated using the Bayesian method with a non-uniform prior distribution of null allele frequencies and prior information on the level of inbreeding (Zhivotovsky 1999). Based on the allele frequencies, the proportion of polymorphic fragments (PLP) at the 5 % level and Nei's gene diversity (*H*) were estimated using AFLP-SURV version 1.0 (Vekemans 2002). To determine whether the intra-population genotypic variation of *B. humilis* was due to somatic mutations, which is the result of vegetative propagation, or recombination, which is associated with the sexual mode of reproduction, the cladistic approach of matrix incompatibility analysis was used (Mes 1998). For binary AFLP data, there are four possible combinations at two loci: 0/0, 0/1, 1/0 and 1/1, and the simplest explanation for the presence of all four combinations in a population is sexual reproduction. The presence of all possible combinations is referred to as 'incompatibility' of individual characters or individuals, because it is very unlikely that all these genotype profiles were produced via somatic mutations. As the incompatibility pattern is generated by recombination, it can be used as a measure of recombination when summed over all pairwise comparisons (Wilkinson 2001). The matrix incompatibility count (MIC) of the *B. humilis* populations was estimated with the help of the JACTAX jackknifing option in PICA version 4.0 (Wilkinson 2001). The MIC was tested with 1,000 permutations using the model of empirical frequencies, and the genotypes with the highest contributions to the MIC were consecutively removed from the total sample until the MIC was zero, which means that genotypes differing only by mutations were left in the matrix. The contribution of many genotypes in the incompatibility matrix is evidence of recent recombination events, and the sequential removal of the genotypes contributing the most to the matrix incompatibility statistics leads to a gradual decrease in the MIC whereas a sharp decline of MIC suggests that there are only a few recombinant genotypes contributing to the incompatibility (Gross et al. 2011).

The presence of loci under selection, i.e. 'outlier' loci that are extremely divergent from the rest of the genome (Luikart et al. 2003), can influence the level of inbreeding (Chybicki et al. 2011), so the hierarchical Bayesian method was performed to detect potential candidate loci using BayeScan software version 2.1 (Foll and Gaggiotti 2008). This method is based on a logistic regression model that divides genetic diversity into population- (β) and locus-specific (α) components; a locus is assumed to be under selection when a locus-specific component is necessary to explain the observed pattern of variation. A positive α value implies diversifying selection, and a negative value may indicate balancing or purifying selection. To calculate a posterior probability for each locus, a reversible-jump Markov Chain Monte Carlo (MCMC) was applied to both models of selection in our dataset, and BayeScan was run by setting the sample size to 10,000 and the thinning interval to 50 (Foll and Gaggiotti 2008). Outliers were identified based on log(Bayes Factor) > 2 and a corresponding posterior probability > 0.99 (Foll and Gaggiotti 2008). Following the removal of outliers, the inbreeding coefficients (f_{AFLP}) were calculated using the FAFLPcalc Excel macro (Dasmahapatra et al. 2007), which is available at http://www.ucl.ac.uk/taxome/kanchon/#publications.

To define the germination capacities of the populations, a median value of the number of germinated seeds per individual (GS) was determined for each locality (Table 2). A two-sample randomization test (test statistic – median difference; 10,000 randomizations) was used to equate the edge and sub-central localities in terms of seed mass, GS, the total number of flowers per flowering individual, and the numbers of female and male flowers per flowering individual. The same sexual reproduction parameters were also compared between the sampling sites with high and low water levels using the two-sample randomization test. A randomization one-way ANOVA (including post hoc tests and Holm's correction; test statistic – median difference; 10,000 randomizations) was carried out to compare seed mass, the total number of flowers per flowering individual, and the numbers of female and male flowers per

flowering individual between populations subject to different degrees of shade (full shade, half shade and lack of shade). To determine which habitat factors (pH, EC, NH_4^+, PO_4^{3-}) were related to the production of inflorescences and seeds, randomized multiple linear regressions (10,000 randomizations) were performed, in which the reproductive efficiency parameters were used as the dependent variables, and the chemical parameters were the independent variables. Within-population medians were used in the multiple regression analyses. Spearman's correlation coefficients were calculated to detect a monotonic relationship between the median values of seed mass and GS as well as between the genetic (PLP, H, f_{AFLP}) and reproductive (median values of the seed mass, the total number of flowers per flowering individual, and the numbers of female and male flowers per flowering individual) parameters. All of the calculations using the habitat and reproductive data were performed with Rundom Pro 3.14 software (Jadwiszczak 2009).

Results

The AFLP analysis included 229 polymorphic loci (70 from *Eco*R1-ACC/*Mse*I-CAC, 73 from *Eco*R1-ACC/*Mse*I-CAT and 86 from *Eco*R1-AGC/*Mse*I-CAC). The error rate was 3.9 %. The proportion of polymorphic fragments (PLP) ranged from 30.1 in the Torfowisko Sobowice (TS) population to 37.9 in Magdzie Bagno (MB), which are both located in Poland (Table 1). The highest Nei's gene diversity value (*H*) was in TS (0.120), and the lowest (0.096) was at the sub-central site, Ozero Borovoe (BOR). The compatibility analysis of each population indicated that most of the genotypic diversity was derived from sexual reproduction; all except 3–4 genotypes contributed to the matrix incompatibility (Fig. 2). The successive removal of the genotypes with the highest contributions generated a gradual decrease in the MIC. For example, in the BOR population (21 individuals studied), MIC reached zero after removing 17 genotypes from the total sample. This means that deleted genotypes contributed the most to the MIC, thus they were formed by recombination events. Only four genotypes in BOR were fully compatible, i.e. they arose as an effect of subsequent mutations. Of the polymorphic loci studied, five were likely under diversifying selection pressure. The inbreeding coefficients were very low in all of the populations (f_{AFLP} = −0.028–0.024; Table 1).

Medians of the reproductive output parameters (the total number of flowers per flowering individual,

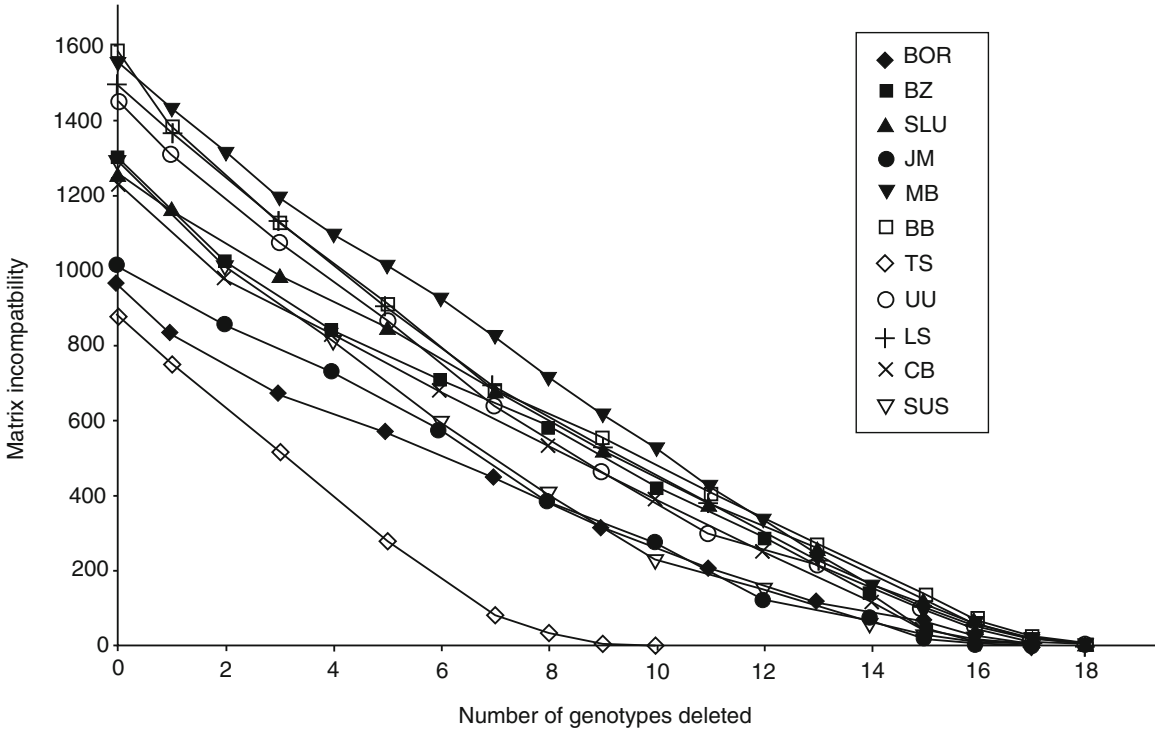

Fig. 2 Graph of character incompatibility. The reduction of the matrix incompatibility count (MIC) corresponds to the successive removal of genotypes from each of the populations studied. Graphic symbols refer to codes of populations described in Table 1.

numbers of female and male flowers per flowering individual, seed mass and number of germinated seeds per individual) for all populations studied are presented in Table 2. The correlation analysis revealed a strongly significant and positive relationship between seed mass and germination capacity ($R_s = 0.573$, $P = 0.000$). There were no statistically significant differences in the total number of flowers per flowering individual, the numbers of female and male flowers per flowering individual and seed mass between edge and sub-central localities, but the number of germinated seeds was substantially higher in Belarus than in Poland (Table 3). None of the results of the Spearman's correlations between the genetic and reproductive parameters were significant (data not shown).

The median values of chemical parameters (pH, EC, NH_4^+, PO_4^{3-}) of habitat quality as well as classification of the populations in respect of groundwater levels and shading are presented in Table 2. The randomization tests revealed that the total number of flowers per flowering specimen and the numbers of male and female flowers per flowering specimen were not influenced by the water level (Table 3). However, seed mass and the number of germinated seeds were significantly higher in wet habitats compared to sites with low water levels. Shading had a statistically significant impact on seed mass, the total number of flowers, and the numbers of female and male flowers per flowering specimen (Table 4). After applying Bonferroni's correction, no significant regression model was found among the four considered for the relationships between the chemical characteristics of the habitat and the reproduction output parameters (Table 5).

Discussion

The nuclear microsatellite analyses demonstrated that the genetic variation among the *Betula humilis* populations in the southwestern and sub-central parts of the species distribution was quite substantial and consistent with the patterns characteristic for outcrossed, wind-pollinated trees and shrubs (Jadwiszczak et al. 2011a,b). Although using the AFLP method, this study did not reveal high values of the genetic variation parameters (PLP = 30.1–37.9; $H = 0.096$–0.120) but confirmed that there was considerable genotypic diversity in the shrub birch at the study locations. Jadwiszczak et al. (2011b) hypothesized that the sexual breeding might explain the maintenance of substantial genetic

Table 3 Results of two-sample randomization tests comparing reproductive parameters (dependent variables) in high (HWL) vs low (LWL) water level habitats and in edge (EP) vs sub-central (SCP) populations (grouping variables).

Variable			Test statistic	*P*
Grouping	Dependent	Medians		
HWL vs LWL	Seed mass	HWL = 0.0177 LWL = 0.0140	0.0037	0.0014*
	Germinated seeds	HWL = 12.0000 LWL = 2.0000	10.0000	0.0024*
	Total No. of flowers	HWL = 13.0000 LWL = 19.0000	−6.0000	0.1791
	Female flowers	HWL = 2.0000 LWL = 3.0000	−1.0000	0.8845
	Male flowers	HWL = 8.0000 LWL = 9.5000	−1.5000	0.3966
EP vs SCP	Seed mass	EP = 0.0155 SCP = 0.0175	−0.0020	0.1278
	Germinated seeds	EP = 3.0000 SCP = 22.0000	−19.0000	0.0001*
	Total No. of flowers	EP = 14.0000 SCP = 15.5000	−1.5000	0.7457
	Female flowers	EP = 9.0000 SCP = 11.0000	−2.0000	0.5691
	Male flowers	EP = 4.0000 SCP = 10.5000	−6.5000	0.0853

*value significant after Bonferroni's correction

Table 4 Results of ANOVA comparing reproductive parameters in unshaded (0), half-shaded (1) and fully shaded (2) populations of *B. humilis*. $P_{adj} - P$ adjusted.

Variable	*d.f.*	*F*	*P*	Post hoc tests
Seed mass	168	14.851	0.0001*	0 vs 2, P_{adj} = 0.0002*
				1 vs 2, P_{adj} = 0.0003*
				0 vs 1, P_{adj} = 0.0262*
Total no. of flowers	180	5.816	0.0039*	1 vs 2, P_{adj} = 0.0087*
				0 vs 2, P_{adj} = 0.0164*
No. of female flowers	180	5.725	0.0037*	0 vs 1, P_{adj} = 0.0015*
No. of male flowers	180	11.400	0.0003*	0 vs 2, P_{adj} = 0.0003*
				0 vs 1, P_{adj} = 0.0058*
				1 vs 2, P_{adj} = 0.0098*

*value significant after Bonferroni's correction

diversity at the population level in the southwestern part of the *B. humilis* range. The matrix incompatibility analysis of the AFLP genotypes seems to support this supposition. The matrix incompatibility count (MIC) revealed that the genotypic diversity of the *B. humilis* stands mainly resulted from recombination because it was necessary to delete many genotypes from the samples to obtain MIC = 0. When incompatibility is a consequence of somatic mutations, the removal of only a few genotypes causes a sharp decrease in the MIC to zero (Gross et al. 2011). The number of genotypes involved in the matrix incompatibility has been found to be higher in fertile populations, compared to the sterile ones, of the threatened shrub *Grevillea rhizomatosa* (Gross et al. 2011). In all of the *B. humilis* samples, the MIC reached zero when only 3–4 genotypes were left, which can mean that the effectiveness of recombination was similar among the

Table 5 Multiple regression analyses of habitat variables on the four parameters of reproductive output of *B. humilis*. All *P* values are statistically non-significant after Bonferroni's correction.

Variable	Coefficient	*t*	*P*	Source	ANOVA			
	B				*d.f.*	*MS*	*F*	*P*
Seed mass (N = 11, R = 0.578, R^2 = 0.000)								
pH	0.0011	0.699	0.503	regression	4	0.000009	0.753	0.595
EC	0.0000	−0.602	0.559	residual	6	0.000012		
NH_4^+	0.0001	0.012	0.990					
PO_4^{3-}	−0.0281	−1.131	0.314					
Total number of flowers per flowering individual (N = 11, R = 0.866, R^2 = 0.582)								
pH	6.7190	0.946	0.382	regression	4	1132.183656	4.489	0.045
EC	−0.0153	−0.604	0.623	residual	6	252.195744		
NH_4^+	−3.4196	−0.132	0.876					
PO_4^{3-}	443.0175	3.883	0.011					
Number of female flowers per flowering individual (N = 11, R = 0.683, R^2 = 0.111)								
pH	1.489	0.728	0.490	regression	4	27.498312	1.313	0.366
EC	0.004	0.616	0.548	residual	6	20.940519		
NH_4^+	11.672	1.568	0.168					
PO_4^{3-}	25.772	0.784	0.479					
Number of male flowers per flowering individual (N = 11, R = 0.872, R^2 = 0.599)								
pH	7.474	1.103	0.287	regression	4	978.650639	4.267	0.026
EC	−0.024	−0.975	0.420	residual	6	229.354120		
NH_4^+	−16.799	−0.682	0.431					
PO_4^{3-}	431.557	3.967	0.008					

endangered shrub birch populations regardless of their location within the species range. Moreover, our analyses demonstrated no statistically significant differences in seed mass, the total number of flowers, and the numbers of female and male flowers per flowering individual between edge and sub-central populations of shrub birch. The results obtained seem to be surprising because it has been suggested that fragmentation and loss of habitats forces plants to allocate more resources to asexual over sexual reproduction, as has been shown in the endangered shrub *Haloragodendron lucasii* (Sydes and Peakall 1998) and the disappearing population of *B. humilis* in northeastern Poland (Chrzanowska and Jadwiszczak 2015). Sexual reproduction was also found to be substantially reduced at marginal localities of the seaweed *Fucus serratus* (Viejo et al. 2011) and the pine *Pinus strobus* (Rajora et al. 2002) compared to centrally located populations. However, both groups of *B. humilis* localities were differentiated with respect to germination capacity (GS), which was significantly higher in sub-central samples compared to edge ones (test statistic = −19.00, $P = 0.0001$). Out of the eight edge populations studied, the GS parameter was very low in five: Bagno Bubnów (BB), TS, Uroczysko Uściwierskie (UU), Łąki Ślesińskie (LS) and Czerwone Bagno (CB), where the median values of GS were not higher than 1.0. The greatest seed germination capacity was observed in the sub-central samples: 32 germinated seeds per specimen in the Sluck (SLU) and 27 in the Berezin'skij Zapovednik (BZ) populations. Although these results are rather average for birches (Holm 1994; Bodył 2006), they may mean that the sexual reproduction of *B. humilis* is more efficient in sub-central localities compared to edge ones. On the other hand, as the germination was tested in laboratory conditions, it is necessary to remember that this parameter of sexual output could be even worse in the natural environment, where the water scarcity, low temperature or granivore pressure can lower the pool of germinated seeds. Perala and Alm (1990) noticed that seed germination of four *Betula* species was generally best at about 20–30°C, temperatures that are rather unusual in Poland and Belarus in early spring.

The small proportion of germinated seeds in some *B. humilis* samples could be a consequence of their low mass, as germination ability was positively correlated with seed mass ($R_s = 0.573$, $P = 0.000$). Seed mass was also significantly correlated with the mean germination rate per population in *B. pendula* in northern Sweden (Holm 1994). A low seed weight may result from the lack of an ovule because birches produce fruits even in the absence of pollination (Atkinson 1992), and in many angiosperm species, empty seeds can also be an effect of self-incompatibility or inbreeding (Wang 2003). Birches are generally recognized as self-incompatible plants (Atkinson 1992) because self-fertilization tests conducted with 31 trees belonging to nine species produced a relatively high proportion of viable seeds only in *B. papyrifera* and *B. alleghaniensis* (Clausen 1966). We believe that inbreeding is not responsible for the production of inviable seeds in *B. humilis*, because the inbreeding coefficient (f_{AFLP}) values in the study populations were very low.

Low seed mass cannot be the only explanation for the poor germination at *B. humilis* sites because the seed weights in the populations SLU and MB were very similar and, at the same time, the number of sprouted seeds per individual was three times higher at SLU. The lack of a difference in the seed weights between the SLU and MB populations could be the result of infections by gall insects of the genus *Semudobia* in MB. Infected *B. pendula* seeds have been shown to be 4–5-fold heavier than healthy seeds (Tylkowski 2012), and gall midges have been responsible for the destruction of up to 18 % of fertilized birch seeds (Atkinson 1992; Holm 1994). Although we did not notice any galls during the selection of seeds for the germination experiment, this explanation for the low number of sprouted seeds in the *B. humilis* populations cannot be excluded.

It is likely that germination of *B. humilis* seeds depends on the environmental conditions. We observed that *B. humilis* seeds were significantly heavier at sites with high water levels compared to populations in dry environments (median difference = 0.004, $P = 0.0014$). As heavier seeds showed better germination ability (median difference = 0.0, $P = 0.0024$), sprouting may be more successful in flooded environments. A germination experiment involving the tree *Vitellaria paradoxa* demonstrated that only the largest seeds in the population retained viability under conditions of water scarcity (Daws et al. 2004). Another habitat factor with a clear impact on the reproductive output of shrub birch was shading. An ANOVA revealed that seed mass decreased significantly in more shaded stands compared to sites with no canopy cover ($F = 14.851$, $P = 0.0001$). Jadwiszczak et al. (2011b) noticed that shrub birches were taller and had a larger leaf surface area in shaded stands compared to shrubs inhabiting sites with open

canopies. This could reflect a life strategy change. To increase fitness, it is likely that birches overgrown by competitive plants allocate more resources to growth and avoid investing in seed production.

However, the significant increase in the total number ($F = 5.816$, $P = 0.0039$) and the numbers of female ($F = 5.725$, $P = 0.0037$) and male ($F = 11.400$, $P = 0.0003$) catkins per flowering individual at more shaded localities compared to unshaded populations seems to contradict the above hypothesis. Why do birches crowded by neighbouring vegetation invest limited resources into the production of flowers? One explanation is that the light-demanding birches start to flower before the canopy becomes too closed, as has been shown in *Arabidopsis thaliana* (Casal 2012). This assumption is questionable, because female birch catkins overwinter as primordia and appear at the time of bud burst, while male inflorescences develop between July and September, but pollen is released during the following spring (Holm 1994). This means that birch flowers are formed during the full growing season. A second possibility is that the excess of male flowers increases the chance of pollination in shaded habitats because pollen dispersal is related to plant height (Sakai and Sakai 2003). In our opinion, the abundance of male flowers is not just an effect of plant size, because all of the individuals we encountered in the CB population were very tall but almost without catkins. A third possibility is that shaded stands of *B. humilis* are, at the same time, rich in phosphorus. Studies conducted in Eurasian wetlands and grasslands have revealed that the investment of plants into sexual reproduction depends significantly on the stoichiometric relationship between nitrogen and phosphorus in the ground (Fujita et al. 2014). In areas with a high N:P ratio (P-limited), individuals start to flower later, have shorter flowering times, fewer seeds and smaller seed masses compared to plants in habitats with a low N:P ratio (N-limited). This is because sexual reproduction is P-consuming, so the abandonment of sexual propagation at P-poor sites allows plants to move the limited resources into growth and defence against pathogens, which increases individual survival (Obeso 2002). However, phosphorus availability does not seem to be a factor responsible for the increase in flower production in shrub birches, because the regression model did not indicate significant relationship between PO_4^{3-} concentrations and the total number of flowers per flowering individual ($F = 4.489$, $P = 0.045$) after Bonferroni's correction. As all results of multiple regression models were non-significant after applying Bonferroni's correction, it might imply that chemical variables of habitat (pH, EC, NH_4^+, PO_4^{3-}) had no influence on the reproductive output of *B. humilis*.

In summary, direct field observations strongly suggest that sexual reproduction in shrub birches can be impaired, especially in edge populations, so how can the pronounced genetic variation at endangered *B. humilis* localities be explained? In our opinion, there are three alternatives. First, birch seeds contain substantial amounts of phosphorus. It has been suggested that the successful recruitment of plants in P-impoverished habitats is due to the production of a few, phosphorus-rich seeds (Lambers et al. 2010). Phosphorus is a key mineral nutrient in every plant process that involves energy transfer, and developing seeds are the main source of phosphorus during plant growth. Lamont and Groom (2013) indicated that young plants developed from large, nutrient-enriched seeds were more protected against drought compared to seedlings sprouted from the smaller seeds. This is because the substantial amounts of phosphorus and nitrogen in large seeds play a critical role in photosynthesis, ensuring sufficient carbon delivery to the rapidly descending roots for effective drought-avoidance. However, supporting a hypothesis about relationship between seed weight and PO_4^{3-} concentrations in *B. humilis* populations would require a more detailed analysis of birch grains. A second explanation is that sexual reproduction is very poor and reflects the current state of the populations, but the estimations of genetic diversity reflect past conditions because the studied genotypes arose some years ago. This supposition can be supported by the lack of significant correlations between genetic diversity and reproductive parameters. Jadwiszczak et al. (2011b) also suggested that the considerable degree of nuclear microsatellite diversity in *B. humilis* stands might be due to insufficient time for a decrease in genetic variation at the southwestern edge of the species' distribution. More than 600 years of the range fragmentation of *Fagus sylvatica* in the Iberian Peninsula resulted in decreased availability of pollen and increased levels of inbreeding and, in consequence, lowered reproduction of trees (Jump and Peñuelas 2006). A third possibility is that the genetic resources of endangered shrub birch populations are enriched by an inflow of genes from the congeners *B. pendula* and *B. pubescens*. Staszkiewicz et al. (1993) suggested that the morphological variation of the leaves, fruits and scales of *B. humilis* could have

arisen from hybridization with common birches. Using cpDNA markers, Jadwiszczak et al. (2012a) demonstrated that sympatric populations of *B. humilis*, *B. pendula* and *B. pubescens* were genetically indistinguishable. Additionally, chromosome analysis conducted in the Polish populations of shrub birch revealed 19–60 % of aneuploid individuals (Jadwiszczak et al. 2011c). Based on the presence of aneuploids in sympatric populations of *B. pendula* and *B. pubescens* (Helms and Jørgensen 1925; Hagman 1971), Jadwiszczak et al. (2011c) suggested that atypical karyotypes of some *B. humilis* specimens originated from interspecific crosses. However, testing the influence of interspecific gene exchange on the genetic diversity of *B. humilis* requires comparative studies of the nuclear genomes of conspecific birches.

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Chapter III

Unfavourable habitat conditions can facilitate hybridisation between the endangered *Betula humilis* and its widespread relatives *B. pendula* and *B. pubescens*

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ARTICLE

Unfavourable habitat conditions can facilitate hybridisation between the endangered *Betula humilis* and its widespread relatives *B. pendula* and *B. pubescens*

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ABSTRACT

Background: Hybridisation can be a threat for the survival of a rare species because, in the case of insufficient numbers of appropriate mates, a rare form is much more likely to cross with a widespread taxon.

Aims: In the present study, we tested hypotheses concerning the level of hybridisation between endangered *Betula humilis* and its widespread congeners: *B. pendula* and *B. pubescens* as a function of habitat conditions.

Methods: We genotyped 312 individuals of three species using AFLP markers. *B. humilis* specimens were sampled in populations with low and high groundwater levels. Morphological identification of *B. pubescens* and *B. pendula* was verified using the Atkinson discriminant function.

Results: Altogether, 15 individuals (4.8%) were indicated as putative hybrids. The *B. humilis* hybrids were found in dry habitats and they could be classified as F1 or F2 generation. Tree hybrids could represent backcrosses to either *B. pendula* or *B. pubescens*.

Conclusions: Genetic analyses contradicted the idea that hybridisation between *B. humilis* and its close relatives was extensive. On the other hand, the presence of introgressed individuals in the populations in areas with low groundwater levels implied that pollen swamping might be a threat for declining *B. humilis* stands.

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AFLP; genetic diversity; habitat deterioration; introgression; marginal populations; pollen swamping

Introduction

Hybridisation is a process of mating and forming viable progeny between individuals from genetically distinct populations of the same species or different species (Rieseberg and Carney 1998; Mallet 2007; Abbott et al. 2013). Detailed studies of 282 families of vascular plants, comprising 3212 genera and species from Europe, North America and Australia revealed that hybridisation was a widespread phenomenon in this group of organisms (Whitney et al. 2010). The evolutionary consequences of interspecific hybridisation can be both positive and negative. Increases in the genetic diversities of both species and populations through the acquisition of neutral or selectively advantageous genes through backcrosses between hybrids and their parental forms (introgression) and the formation of new species are undoubtedly positive effects of hybridisation (Mallet 2007; Abbott et al. 2013). However, disruption of co-adapted genes and blurring or extinction of a local gene pool are negative repercussions of interspecific mixing (Largiadèr 2007). Hybridisation may be disadvantageous for rare species and at range margins because, in the case of insufficient numbers of appropriate mates, the less common forms are likely to cross with widespread taxa (Barton and Hewitt 1985; Rhymer and Simberloff 1996). For example, the rare Sicilian endemic *Calendula maritima* Guss. was put at risk of extinction as a result of gene inflow from the more common congener *C. suffruticosa* subsp. *fulgida* (Raf.) Guadagno (Plume et al. 2015).

Among vascular plants, the *Betula* (birch) genus has been recognised as one of the taxa most involved in hybridisation (Whitney et al. 2010; Barrington 2011). The genus includes monoecious, wind-pollinated and wind-dispersed trees and shrubs inhabiting various ecosystems in the temperate, boreal and arctic climate zones of Eurasia and North America. In Europe, there are two tree birches: *Betula pendula* Roth and *Betula pubescens* Ehrh. and two shrub forms: *Betula nana* L. and *Betula humilis* Schrk. Some studies have evidenced that the extent of admixture between sympatric birches varies with latitude, being higher in the northern parts of the Eurasian continent than in

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other areas (Kallio et al. 1983; Wang et al. 2014a; Zohren et al. 2016; Tsuda et al. 2017). According to Kallio et al. (1983), such phenomenon could be a consequence of overlapping of flowering seasons among *Betula* congeners inhabiting the subarctic regions. Increasing frequencies of the nuclear microsatellite and RAD alleles of *B. nana* into *B. pubescens* from the southern to northern parts of Great Britain could also be explained by a historical decline and/or northwards shift in the range of *B. nana* during Holocene warming (Wang et al. 2014a; Zohren et al. 2016). A high frequency of abnormal hybrid pollen grains that was found in an early Holocene peat profile in Iceland confirmed that interspecific gene flow between sympatric *Betula* species was possible in suitable climatic and ecological conditions (Karlsdóttir et al. 2009).

The shrub birch *B. humilis* is the only birch species that always exists in sympatric populations with close congeners; thus, it is potentially highly involved in hybridisation. *Betula humilis* forms abundant populations in Siberia, north-western Mongolia and eastern Europe, but it is endangered (EN category, IUCN) in the western and central parts of the European continent. The distribution range of the species reaches its south-western margin in Poland, where approximately 80% of its stands disappeared during the twentieth century (Jabłońska 2014; Załuski et al. 2014). *Betula humilis* is a poor competitor in dry regions and stands with high concentrations of calcium ions (Jabłońska 2012); hence, the main causes of its disappearance are lowering of groundwater levels and declines in the use of wet meadows. Additionally, pollen swamping from common *B. pendula* and *B. pubescens* can threaten declining populations of the shrub birch (Staszkiewicz et al. 1993).

Until recently, few investigations were conducted to evaluate the gene exchange between diploid ($2n = 28$) *B. humilis*, diploid *B. pendula* ($2n = 28$) and tetraploid ($2n = 56$) *B. pubescens*. Chloroplast DNA (cpDNA) analysis has suggested extensive haplotype sharing among the three species in Polish and Belarusian populations (Jadwiszczak et al. 2012). Karyological investigations have revealed up to 60% of aneuploid individuals ($2n \neq 28$) in six Polish stands, and hybridisation was proposed as one of the possible explanations of this phenomenon (Jadwiszczak et al. 2011b). Conversely, Natho (1959) has found that putative morphological hybrids in German populations had chromosome numbers typical for one parental taxon. Based on the morphology of leaves, scales and nutlets of 60 individuals in 12 populations, Staszkiewicz et al. (1993) have suggested that specimens with larger leaves, which constituted ca. 45% of studied samples, could be hybrids and introgressed forms. Thus, hybridisation could be accelerating the disappearance of populations of the species. Our study tested, using AFLP (amplified fragment length polymorphism) molecular markers, if (1) there were significant contributions of mixed individuals to extant *B. humilis* populations (Staszkiewicz et al. 1993), (2) genetic exchange between the tree birches and *B. humilis* was facilitated in deteriorated habitats compared to undisturbed localities (Staszkiewicz et al. 1993), and if (3) *B. humilis* hybridised with *B. pubescens* more frequently in wet habitats and more frequently with *B. pendula* in dry habitats (Natho 1959).

Material and methods

Sample collection

Studies were conducted in northern, north-eastern and south-eastern Poland (Figure 1). Altogether, 157 *B. humilis* individuals were analysed across eight populations, 76 specimens of *B. pendula* were analysed across seven populations, and 79 *B. pubescens* individuals were analysed across seven populations (Table 1). The *B. humilis* specimens used were the same as those described by Chrzanowska et al. (2016). The specimens were collected from populations with high (≥ -10 cm) and low (≤ -10 cm) groundwater levels because Jabłońska (2012) has noticed that the shrub birch preferred habitats with water levels reaching the soil surface.

For DNA isolation, young leaves of morphologically pure tree birches were collected. To minimise the chance of collecting leaves from the vegetative ramets, a minimum distance of 50 m in the tree birch stands was arbitrarily implemented. Fresh leaves were kept in a refrigerator until they were transferred to the laboratory and then stored at −80°C. For each *Betula* tree with at least a few short shoots with leaves, the second leaf was sampled, and its photograph was taken in order to collect measurements and calculate the Atkinson discriminant function (ADF; Atkinson and Codling 1986). This function allows *B. pendula* and *B. pubescens* to be discriminated based on leaf shape. All specimens with ADF ≥ -2 were identified as *B. pendula*, those with ADF < -2 were recognised as *B. pubescens* (Wang et al. 2014b). Five leaves per individual were measured. The ADF was calculated in 76 individuals of *B. pendula* and 63 of *B. pubescens*.

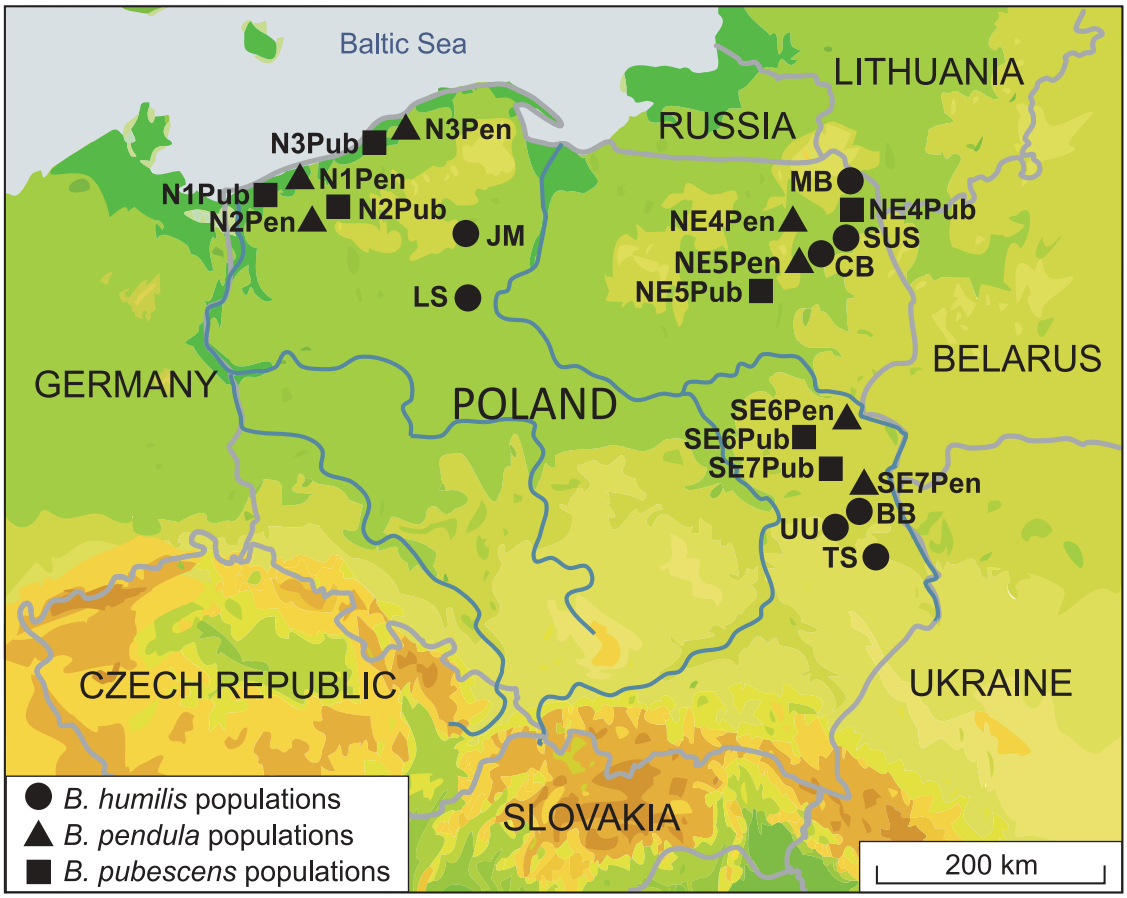

Figure 1. Location of *B. humilis, B. pendula* and *B. pubescens* populations in Poland. Population codes according to Table 1.

Table 1. Locality information and genetic diversity parameters of *B. humilis, B. pendula* and *B. pubescens* sampled, Poland. Water level: H – high (≤ 10 cm), L – low (≥ 10 cm), *data after Chrzanowska et al. (2016); *N* – numbers of sampled individuals, *PLP* – percentage of polymorphic loci, H_S – expected heterozygosity.

			Location					
Region	Species	Population code	Latitude	Longitude	Water level*	*N*	*PLP*	H_S
Northern Poland	*B. humilis*	JM	53°47′N	17°46′E	H	20	29.2	0.081
		LS	53°08′N	17°41′E	L	21	37.0	0.097
	B. pendula	N1Pen	54°08′N	15°19′E	-	8	30.1	0.111
		N2Pen	53°48′N	15°31′E	-	12	30.2	0.092
		N3Pen	54°34′N	16°46′E	-	6	26.6	0.102
	B. pubescens	N1Pub	54°03′N	14°59′E	-	13	29.8	0.088
		N2Pub	53°52′N	15°44′E	-	5	29.2	0.120
		N3Pub	54°31′N	16°30′E	-	9	27.9	0.089
North-eastern Poland	*B. humilis*	MB	54°08′N	23°16′E	H	21	35.1	0.092
		SUS	53°43′N	23°01′E	H	20	37.4	0.093
		CB	53°37′N	22°49′E	L	20	34.3	0.083
	B. pendula	NE4Pen	53°46′N	22°24′E	-	13	27.6	0.077
		NE5Pen	53°32′N	22°35′E	-	11	28.7	0.094
	B. pubescens	NE4Pub	53°48′N	23°17′E	-	11	31.1	0.094
		NE5Pub	53°16′N	21°51′E	-	17	32.9	0.083
South-eastern Poland	*B. humilis*	BB	51°22′N	23°16′E	L	21	39.8	0.099
		UU	51°21′N	23°03′E	L	21	36.8	0.091
		TS	51°07′N	23°23′E	L	13	32.4	0.098
	B. pendula	SE6Pen	52°07′N	23°10′E	-	16	40.0	0.109
		SE7Pen	51°37′N	23°19′E	-	10	30.1	0.096
	B. pubescens	SE6Pub	52°02′N	22°25′E	-	8	31.0	0.105
		SE7Pub	51°43′N	22°54′E	-	16	33.2	0.092

Laboratory analyses

Leaves were homogenised in a TissueLyser LT bead mill (Qiagen), and then DNA was extracted with the AX Plant Kit (A&A Biotechnology) according to the manufacturer's instructions. Total DNA was used for AFLP genotyping following the procedure of Vos et al. (1995) with some modifications, suggested by Applied Biosystems (AFLP Plant Mapping Protocol). AFLP genotypes were established based on three primer combinations: *Eco*RI-ACC/*Mse*I-CAC, *Eco*RI-ACC/

*Mse*I-CAT and *Eco*RI-AGC/*Mse*I-CAT. The first and second primer combinations were the same as those described in Chrzanowska et al. (2016) for *B. humilis*; thus, the genotypes of only tree birches were prepared from the restriction-ligation stage and were combined with ready *B. humilis* profiles. The *Eco*RI-AGC/*Mse*I-CAT combination was new for all specimens used in this study and was chosen after testing 20 combinations. An ABI PRISM 3130 (Applied Biosystems) sequencer with a GeneScan 500 LIZ Size Standard (Applied Biosystems) was used to separate the NED-labelled products of the selective amplifications. AFLP profiles were analysed using GENEMAPPER 4.0 (Applied Biosystems) including fragments (≥100 RFU) between 70 and 500 bp. In addition, one individual of *B. pendula* and one of *B. pubescens* from each population were replicated for the three primer combinations starting from the restriction-ligation reaction to calculate the error rate according to the method of Bonin et al. (2004).

Statistical analyses

Our data consisted of both diploid and tetraploid species; thus, the software package GenoDive 2.0b23 (Meirmans and Van Tienderen 2004) was used as it comprises a correction for missing dosage information for polyploids in multiple analyses. In this way, the assignment of individuals to clones was checked, and Nei's gene diversity (H_S) in each population was calculated. The percentage of polymorphic loci (*PLP*) in each population was estimated using GeneAlEx 6.5 (Peakall and Smouse 2006, 2012). The *PLP* and H_S parameters were corrected for small sample sizes using the formula in Nei and Chesser (1983).

To establish the most likely number of independent genetic populations, the Bayesian cluster analysis in STRUCTURE v2.3.4 (Pritchard et al. 2000), with the recessive allele model implemented for analyses of polyploids and dominant data (Falush et al. 2007) as well as with and without the LOCPRIOR model (locality information) for the clustering assistance (Hubisz et al. 2009), was utilised. The input file was created according to the method of Paule et al. (2015). This meant that each specimen was coded as a formal tetraploid with missing chromosome sets assigned as 'missing data' in the case of both diploid *B. pendula* and *B. humilis*. An admixture model and correlated allele frequencies were chosen, and 10 independent runs examining the number of clusters (between K1 and K22) with 500,000 iterations after 50,000 burn-in periods were carried out. Next, the optimal value of K was inferred from the ad hoc statistic ΔK (Evanno et al. 2005), using the online software STRUCTURE HARVESTER v0.6.93 (Earl and von Holdt 2012). To examine the genetic relationship between individuals within and between the three birch species, principal coordinates analysis (PCoA) implemented in PAleontological STatistics (PAST) version 3.14 (Hammer et al. 2001) was carried out on the matrix of pairwise Jaccard's genetic similarity coefficients, as it was suggested by Kosman and Leonard (2005). Pair-wise genetic differentiation between birch species was estimated using the ρ (Rho) statistic (Ronfort et al. 1998), which is independent of the rate of double reduction and comparable between ploidy levels (Meirmans et al. 2018). Moreover, we also calculated the G''_{ST} parameter, which is an unbiased estimator of F'_{ST} (Meirmans and Hedrick 2011). Both ρ and G''_{ST} were calculated in GenoDive.

Identification of potential interspecific hybrids was conducted with the help of STRUCTURE, the R package '*parallelnewhybrid*' (Wringe et al. 2017) and GenoDive. First, for K = 2, the mean value of the cluster membership coefficient (Q) was calculated for each individual across five runs in STRUCTURE using the LOCPRIOR model, and these measures were used as hybrid indices (Devitt et al. 2011; Fogelqvist et al. 2015). Any individual with a Q value between 0.1 and 0.9 was considered to be of a mixed origin, while the pure class (*B. humilis* vs. *B. pendula*/*B. pubescens*) genotypes were characterised by Q values ≥ 0.9. The Bayesian posterior probabilities computed in '*parallelnewhybrid*' were used to indicate the membership of each individual to any of the different categories (Anderson and Thompson 2002): pure classes, F1 (hybrid between *B. humilis* and *B. pendula* or *B. pubescens*), F2 and two categories of backcrosses (F1 × *B. humilis* and F1 × tree birch parent). The default genotype categories for first and second generations of crossing were applied (Anderson and Thompson 2002). Five runs of the MCMC based on 100,000 iterations after a burn-in of 10,000 iterations were conducted to estimate the hybrid class assignment accuracy. The method of Buerkle (2005) implemented in GenoDive was used to carry out the last hybrid analysis. As this software requires definitions for both pure parental forms and mixed individuals, the hybrid class consisted of specimens indicated by STRUCTURE and/or '*parallelnewhybrid*' as being of mixed origin, while the remaining individuals were classified as *B. humilis* or *B. pendula*/*B. pubescens*.

Results

The Atkinson's function (Atkinson and Codling 1986) revealed that three *B. pendula* individuals were characterised by the ADF values (−17.26, −3.72 and −14.74) specific to *B. pubescens*, while two *B. pubescens* specimens had ADF values (6.4 and −1.34) typical for *B. pendula* (the data for only some pure and mixed individuals are shown in Table 2 and Figure 2). The highest ADF value was 36.60, while the lowest was −32.90. Based on the ADF, the dataset for genetic analyses was corrected by 'allocation' of five tree specimens between populations within the region.

The genetic analyses included 491 polymorphic AFLP loci: 102 loci from *Eco*RI-ACC/*Mse*I-CAC, 257 from *Eco*RI-ACC/*Mse*I-CAT, and 132 from *Eco*RI-AGC/*Mse*I-CAT. The error rate was 3.7%. The contribution of polymorphic loci ranged from *PLP* = 26.6 in N3Pen to *PLP* = 40.0 in the SE6Pen population, and Nei's gene diversity was the lowest in NE4Pen (H_S = 0.077) and highest (H_S = 0.120) in N2Pub (Table 1).

Genetic differentiation, which was calculated as the ρ and G''_{ST} parameters, gave very similar results (Table 3). The highest values of both statistics were noticed between *B. humilis* and the two tree birches.

Table 2. Identification of hybrid individuals in the *B. humilis* (*B. hum*), *B. pendula* and *B. pubescens* (*B. pen/B. pub*) populations based on the *Q* values in STRUCTURE, posterior probabilities in *'parallelnewhybrid'*, hybrid index in GenoDive and leaf morphology (ADF), Poland. ID – individual number, BC – backcross, na – leaves not available.

		STRUCTURE			*Parallelnewhybrid*							
		Q values										
	ID	*B. hum*	*B. pen/ B. pub*	Category	Pure *B. hum*	Pure *B. pen/ B. pub*	F1	F2	*B. hum* BC	*B. pen/ B. pub* BC	GenoDive hybrid index	ADF
1	LS13	0.63	0.37	mixed	1.00	0.00	0.00	0.00	0.00	0.00	0.60	-
2	BB8	0.59	0.41	mixed	1.00	0.00	0.00	0.00	0.00	0.00	0.55	-
3	TS1	0.72	0.28	mixed	0.90	0.00	0.00	0.02	0.08	0.00	0.69	-
4	N2Pen12	0.15	0.85	mixed	0.00	0.01	0.00	0.00	0.00	0.99	0.19	17.42
5	N3Pen7	0.11	0.89	mixed	0.00	0.70	0.00	0.00	0.00	0.30	0.16	20.44
6	NE4Pen1	0.06	0.94	pure B. *pen/B. pub*	0.00	0.75	0.00	0.00	0.00	0.25	0.17	17.90
7	NE4Pen3	0.07	0.93	pure B. *pen/B. pub*	0.00	0.73	0.00	0.00	0.00	0.27	0.14	23.54
8	NE4Pen10	0.08	0.92	pure B. *pen/B. pub*	0.00	0.65	0.00	0.00	0.00	0.35	0.17	19.20
9	SE6Pen11	0.12	0.88	mixed	0.00	0.92	0.00	0.00	0.00	0.08	0.14	16.52
10	N1Pub7	0.02	0.98	pure B. *pen/B. pub*	0.00	0.78	0.00	0.00	0.00	0.22	0.06	na
11	N2Pub1	0.08	0.92	pure B. *pen/B. pub*	0.00	0.42	0.00	0.00	0.00	0.58	0.11	−6.78
12	NE4Pub2	0.07	0.93	pure B. *pen/B. pub*	0.00	0.34	0.00	0.00	0.00	0.66	0.13	na
13	NE4Pub7	0.08	0.92	pure B. *pen/B. pub*	0.00	0.15	0.00	0.00	0.00	0.85	0.13	na
14	NE4Pub8	0.06	0.94	pure B. *pen/B. pub*	0.00	0.35	0.00	0.00	0.00	0.65	0.12	−18.58
15	NE5Pub5	0.06	0.94	pure B. *pen/B. pub*	0.00	0.65	0.00	0.0	0.00	0.35	0.07	−8.74

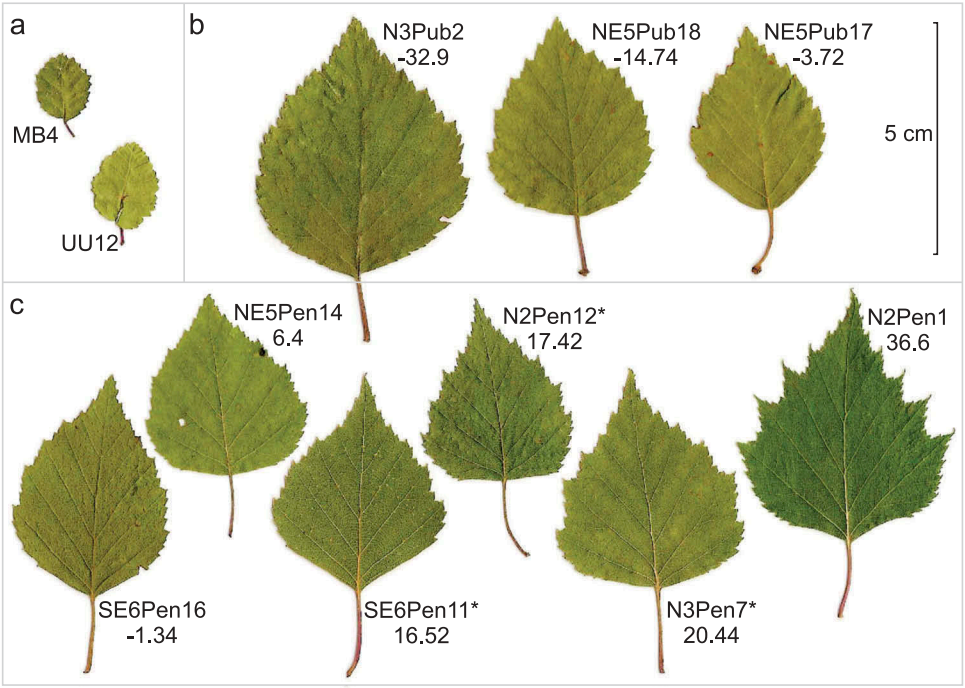

Figure 2. Leaf morphology of selected individuals of (a) *B. humilis*, (b) *B. pubescens* and (c) *B. pendula*. The ADF values of tree birches are shown under their individual numbers. Potential tree hybrids defined by STRUCTURE are marked with an asterisk.

Table 3. Pair-wise ρ (above diagonal) and G''_{ST} (below diagonal) among *Betula* species, Poland.

	B. humilis	*B. pendula*	*B. pubescens*
B. humilis	-	0.344	0.333
B. pendula	0.391	-	0.072
B. pubescens	0.381	0.087	-

Genetic differentiation between *B. pendula* and *B. pubescens* was several times lower (ρ = 0.072, G''_{ST} = 0.087). The STRUCTURE examination revealed two genetic groups in both analyses running with and without LOCPRIOR option; hence, only data with locality information are shown (Figure 3(a)). The first cluster consisted of *B. humilis* populations, while *B. pendula* and *B. pubescens* formed the other group. Neither the three (Figure 3(b)) nor five (Figure 3(c)) genetic clusters showed clear delimitation of the tree birches, and almost all specimens represented a mixture of at least two diverse genetic backgrounds, while the *B. humilis* populations were always distinct. PCoA confirmed that *B. humilis* individuals were grouped separately from the two tree birches (Figure 4(a)). *Betula pendula* and *B. pubescens* were intermixed when the first (22.6% of the total variance explained) and second (5.5%) axes were considered. A quite clear separation of all three congeners was observed when the first and third (2.8%) axes were examined (Figure 4(b)).

Altogether, 15 individuals (4.8%) were indicated as being of mixed origin by STRUCTURE and/or GenoDive (Table 2). Based on a 0.1 – 0.9 range of *Q* values estimated by STRUCTURE, three *B. humilis* and three *B. pendula* individuals were inferred to be of hybrid origin. Considering the *B. humilis* populations, single mixed specimens were sampled in LS, BB and TS. All these populations were characterised by low groundwater levels (Table 1). Among the mixed individuals, only LS13, BB8 and TS1 could possibly represent the F1 or F2 generation because their *Q*-values were 0.63(0.37), 0.59(0.41) and 0.72(0.28), respectively.

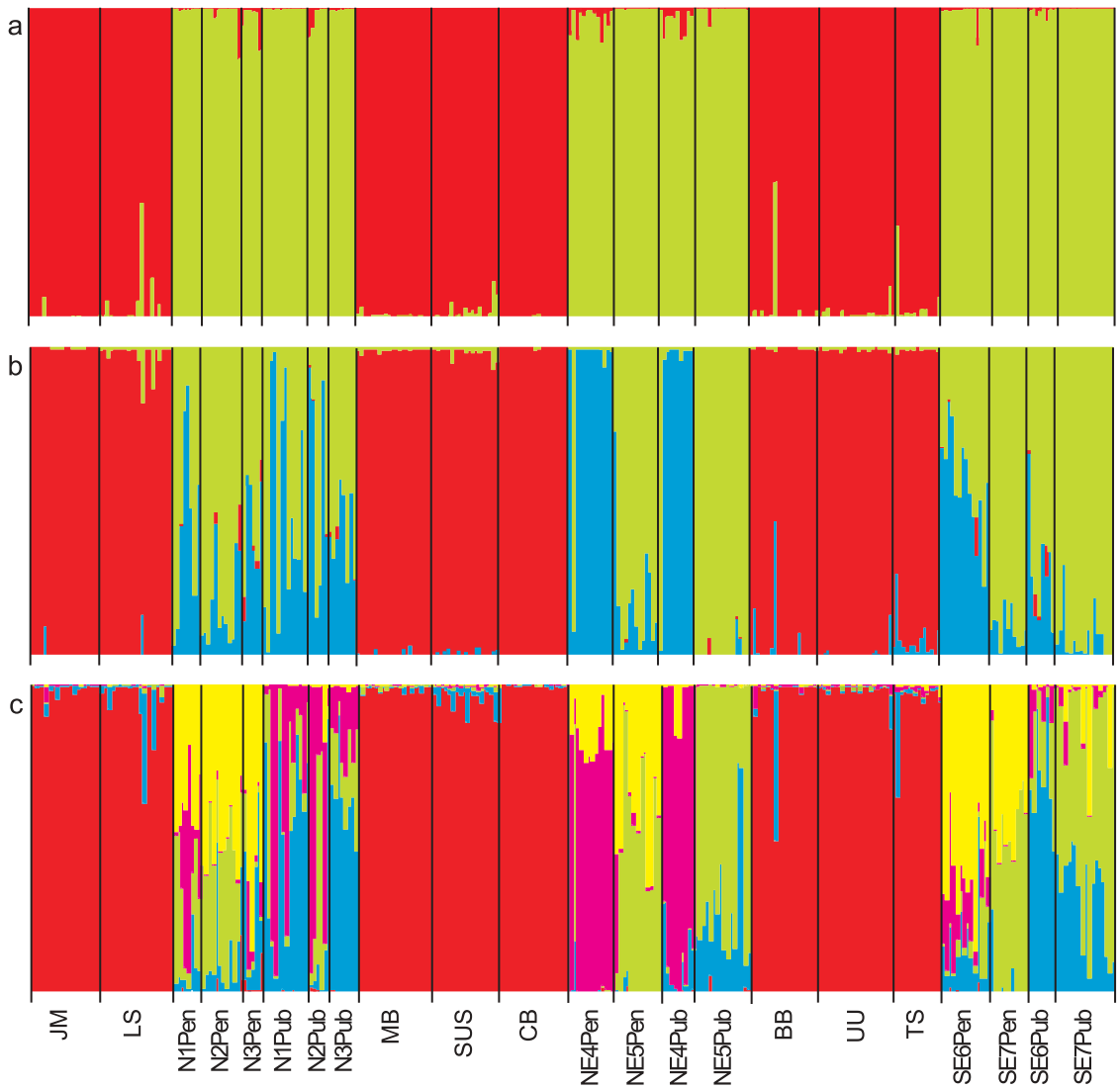

Figure 3. Estimated genetic admixture of *B. humilis, B. pendula* and *B. pubescens* at AFLP loci with (a) *K* = 2, (b) *K* = 3 and (c) K = 5. Population codes according to Table 1.

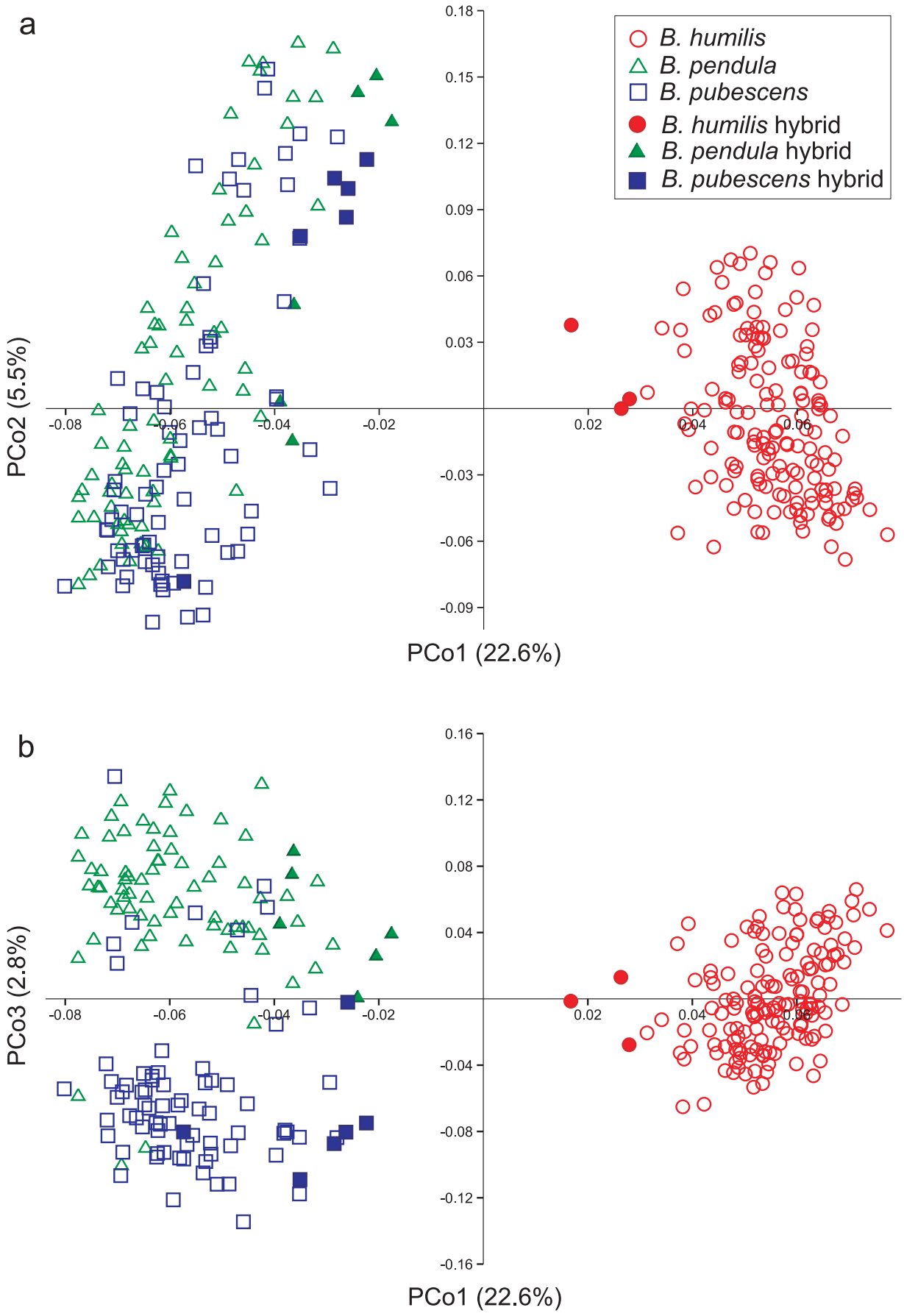

Figure 4. Principal Coordinates Analysis (PCoA) revealing the Jaccard's genetic distances between individuals of *B. humilis, B. pendula* and *B. pubescens* according to: (a) PCo1 and PCo2 axes, (b) PCo1 and PCo3 axes.

All remaining potential hybrids could be classified as backcrosses of tree birches. According to *'parallelnewhybrid'*, all *B. humilis* specimens were pure species with posterior probabilities from 0.9 to 1.0. Among the tree birches, six *B. pubescens* and five *B. pendula* individuals revealed increased probability (0.22–0.99) to be backcrosses to the tree species. The hybrid index values revealed by GenoDive ranged from 0.06 (N1Pub7) to 0.69 (TS1) and the hybrid origin of the *B. humilis* individuals indicated by STRUCTURE was confirmed by the GenoDive analysis. BB8 individual reached average hybrid index value of 0.55. Leaf measurements of the available tree hybrid samples showed typical ADF values for *B. pendula* or *B. pubescens* (Table 2; Figure 2).

Discussion

Our analyses, involving 312 individuals of three birch species genotyped at 491 AFLP loci, revealed a low level of genetic admixture between endangered *B. humilis* and its widespread congeners *B. pendula* and *B. pubescens*. In the eight populations of *B. humilis*, only three (1.9%) individuals were characterised by *Q* values and/or hybrid index values less than 0.9. They were classified as putative F1s or F2s based on the average *Q* values and hybrid indices. This finding excludes that the considerable number of aneuploids in the shrub birch stands (Jadwiszczak et al. 2011b) results from interspecific hybridisation. The statement by Staszkiewicz et al. (1993), implying that approximately 45% of the *B. humilis* individuals could be hybrid and introgressed forms, was not confirmed, either. Staszkiewicz et al. (1993) have analysed three leaf characters, scale types and nutlet sizes, giving them scores from 0 to 3 or 4; next, the scores were combined into a single value. Values within the range 2–5 were described as typical for *B. humilis*, while values from 6 to 10 were suggested to indicate introgression from tree birches. The values characteristic of *B. pendula*/*B. pubescens* were assessed to range from 12 to 15. As 26 out of the 60 individuals studied by these authors showed high morphological values, it was concluded that the contributions of hybrids in the *B. humilis* populations were substantial. However, the genetic data are in agreement with our field experience. We observed that *B. humilis* individuals with intermediate morphology, which could strongly suggest their mixed origin, were very scarce. Over more than 10 years of investigations, we observed such two specimens, one in the Rospuda Valley in Poland and one in the Berezin'skij Zapovednik in Belarus (Jadwiszczak; pers. obs.). None of these localities were sampled in this study. Staszkiewicz et al. (1993) have collected the leaves from herbarium specimens; thus, they possibly did not consider the impact of sunlight availability on the morphology of shrub birches. Some authors have noticed that the shrub birches are much taller and have bigger leaves in coppice stands than in the unshaded habitats (Jadwiszczak et al. 2011a; Załuski et al. 2014; Chrzanowska et al. 2016). To study the influence of habitat and genotype on the shape and size of *B. humilis* leaves, Jabłońska (2009) has carried out an experiment with cuttings obtained from four different genetic individuals. Each genotype was planted in four different treatments: (1) dry mire soil – sunlit, (2) dry mire soil – shaded, (3) wet peat – sunlit and (4) wet peat – shaded. Leaves of clones grown on the shaded, dry mire soil were significantly larger than those grown on other sites. The differences in leaf shape characters were mostly non-significant (Jabłońska 2009). It was evidenced that the shrubs *Spiraea alba* Du Roi and *S. tomentosa* L. also responded to shade by increasing both leaf area and specific leaf area in a greenhouse experiment (Stanton et al. 2010).

The three potential hybrids in the *B. humilis* populations were found in locations characterised by low water table: LS, BB and TS (Jadwiszczak et al. 2015; Chrzanowska et al. 2016). Although the number of hybrids is very low, our results can imply that gene inflow from common congeners may be more frequent in *B. humilis* in drained locations with low groundwater levels rather than in undisturbed populations (Staszkiewicz et al. 1993). Such habitat dependent hybridisation can be a consequence of pollen swamping and/or change in selection pressure. It has been suggested that higher levels of hybridisation in small or marginal populations could result from weak selection pressure acting against mixed genotypes (Rieseberg 1997). In Iceland, hybrids between *B. nana* and *B. pubescens* are often found at population edges, which may imply that they are selectively inferior compared to pure species individuals (Anamthawat-Jónsson and Thórsson 2003). It is likely that individuals with an admixture of *B. pendula* or *B. pubescens* genes gain a selective advantage in habitat conditions in which pure *B. humilis* individuals have diminished competitive abilities.

The discovery of potential hybrids in the disappearing populations of *B. humilis* may suggest that pollen swamping can threaten the endangered birch species in adverse habitat conditions. If reproductive barriers are not complete, the less

abundant species are likely to produce more hybrid seeds than widespread congeners due to pollen swamping (Nagamitsu et al. 2006). This difference is because specimens in small populations are much more likely to breed with common congeners because of the difficulty in finding mates of the same species (Barton and Hewitt 1985; Rhymer and Simberloff 1996). Pollen swamping has been indicated as a cause of the displacement of hexaploid populations of the wind-pollinated plant *Mercurialis annua* L. by the diploid form in some parts of northern Spain (Buggs and Pannell 2006). No study has suggested full reproductive barriers between European birches so far. Depending on the marker used, interspecific gene flow within the *Betula* genus has been reported to be substantial (Palmé et al. 2004; Maliouchenko et al. 2007; Thórsson et al. 2010; Jadwiszczak et al. 2012; Wang et al. 2013) or negligible (Wang et al. 2014a; Eidesen et al. 2015; Tsuda et al. 2017). The presence of hybrids in the *B. humilis* locations as well as the admixture of *B. humilis* genes in the genetic pools of *B. pendula* and *B. pubescens* also indicate that the barriers of reproductive isolation are not complete between the investigated species. It has been suggested that introgression is more likely from a diploid into tetraploid species as opposed to the reciprocal direction in the *Betula* genus (Stebbins 1971; De Groot et al. 1997). This means that the effectiveness of interspecific hybridisation should be greater when *B. pubescens* is the male parent, and diploid *B. humilis* or *B. pendula* is the female parent than vice versa. One possible explanation is that larger pollen grains of *B. pubescens* produce more pollen tube growth compared to the pollen tubes of diploid species (Anamthawat-Jónsson and Tómmason 1999).

We failed to test Natho's (1959) hypothesis that *B. humilis* crosses with *B. pendula* mainly in dry habitats and with *B. pubescens* at the wet sites. Firstly, we found the three putative hybrids only in the *B. humilis* stands. All of them came from the shrub birch populations inhabiting locations with low groundwater levels. Additionally, the genetic pools of *B. pendula* and *B. pubescens* were not distinguished by STRUCTURE. Individuals of *B. pendula* and *B. pubescens* were also intermixed in PCoA when the first and second axes were considered, while *B. humilis* was clearly different from the two tree birches. Consideration of the first and third axes allowed to separate most of the *B. pendula* and *B. pubescens* specimens. Some authors have emphasised, however, that one should be careful when interpreting the dissimilarity metrics calculated for different ploidy levels and unknown allele dosage (Kosman and Leonard 2005; Meirmans et al. 2018). It is likely that a low-resolution power of the AFLPs was a main reason of discrimination failure in the present study, although these markers were successfully applied to discriminate three birch species: tetraploid *B. pubescens*, diploid *B. nana* and diploid *B. glandulosa* L. (Eidesen et al. 2015). *Betula pendula* and *B. pubescens* have been found clearly distinct from one another in the analyses based on the nuclear microsatellites (Wang et al. 2014a; Zohren et al. 2016; Tsuda et al. 2017).

We found weaker genetic differentiation (ρ = 0.072; G''_{ST} = 0.087) between tree congeners compared to differentiation between the shrub birch and *B. pendula* (ρ = 0.344; G''_{ST} = 0.391) or *B. pubescens* (ρ = 0.333; G''_{ST} = 0.381). Comparing species with different ploidy levels is a great challenge because many factors can influence their genetic diversities (see Meirmans et al. 2018). Tsuda et al. (2017) have studied six birch species across Eurasia and suggested that biparentally inherited nuclear DNA was much more effective at discriminating particular birch taxa than uniparentally inherited cpDNA. Considering nuclear markers only, the RAD tag loci that were more abundant in genomes showed sharper resolution of *B. pendula* and *B. pubescens* than the 12 nuclear microsatellites (Wang et al. 2014a; Zohren et al. 2016). A RAD loci analysis conducted in British birch populations suggested that gene flow was higher between tree congeners than between shrub and tree forms (Zohren et al. 2016). The results obtained by Tsuda et al. (2017) have shown that this was not always the case, as the F'_{ST} value between *B. pubescens* and *B. pendula* was 0.491, while this parameter was much lower (0.285) between *B. pubescens* and *B. nana*. It is worthy to underline, however, that Tsuda et al. (2017) have considered much greater area of the birches ranges than it was done in our investigation.

One can suppose that the gene exchange between tree congeners can be responsible for their morphological similarities. We also misidentified the three *B. pendula* and two *B. pubescens* individuals in the field, which was revealed by the calculations of the ADF (Atkinson and Codling 1986). Two out of the five specimens were characterised by ADF scores of −3.72 and 1.34, which were close to the threshold of −2 (Wang et al. 2014b). However, the potential tree hybrids had ADF scores typical for a pure species and they

were mostly placed together with pure individuals of the proper species in PCoA when the first and third axes were considered. In Great Britain, the majority of birches with genetic admixture at the nuclear microsatellite loci also had ADF scores characteristic of one of the parental taxa; hence, hybridisation seems not to be the main cause of the morphological continuum between *B. pendula* and *B. pubescens* (Wang et al. 2014b).

It has been suggested that considerable genetic diversity in the *B. humilis* populations situated at the south-western edge of its geographic range could be an effect of introgression from *B. pendula* and/or *B. pubescens* (Jadwiszczak et al. 2011a; Chrzanowska et al. 2016). AFLP analysis revealed that the parameters of genetic variation (*PLP* = 29.2 – 39.8; H_S = 0.081 – 0.099) in the shrub birch localities were comparable to those found in *B. pubescens* (*PLP* = 27.9 – 33.2; H_S = 0.083 – 0.120) and *B. pendula* (*PLP* = 26.6 – 40.0; H_S = 0.077 – 0.111); thus, the endangered *B. humilis* does not seem to be genetically depauperate. However, as only few potential hybrids were detected, the supposition that gene inflow from the tree congeners can increase the genetic diversity of the shrub birch is unlikely.

Conclusions

The aim of the present study was to examine the level of hybridisation between endangered *B. humilis* and its widespread congeners: *B. pendula* and *B. pubescens* in the locations with low and high water tables. Results obtained in this study imply that hybridisation between *B. humilis* and tree birches appears to be more likely in dry habitats than in the stands with high groundwater levels. This finding is striking because one of the main causes of *B. humilis* decline is lowering of groundwater levels and, in consequence, the colonisation of its habitats by forest and brushwood plants (Załuski et al. 2014). However, small number of detected individuals with potential hybrid origin in the *B. humilis* populations contradicts the hypotheses of Staszkiewicz et al. (1993) and Jadwiszczak et al. (2011b) about widespread mixing with birch congeners. Thus, suggestions of Jadwiszczak et al. (2011a) and Chrzanowska et al. (2016), that considerable genetic diversity of *B. humilis* at the margin of its range can result from genetic introgression from close congeners, have to be rejected. As effective sexual reproduction has not been confirmed, either (Chrzanowska et al. 2016), a reason of considerable genetic diversity at the species range margin remains still unexplained. Therefore, the hypothesis suggesting the presence of few, but phosphorus-rich seeds, which can enable successful plant development in the *B. humilis* sites (Chrzanowska et al. 2016), should be tested in the future.

Disclosure statement

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Chapter IV

Clonal diversity, gene flow and seed production in endangered populations of *Betula humilis* Schrk.

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ORIGINAL ARTICLE

Clonal diversity, gene flow and seed production in endangered populations of *Betula humilis* Schrk.

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Abstract

Many plant species can reproduce by both sexual and vegetative means. Clonal diversity and degree of intermingling of clones in the vegetative reproductive mode can influence the mating and fertility of individuals. The aim of the study was to assess the clonal structure and its potential influence on gene flow and generative reproduction efficiency in six endangered *Betula humilis* populations from the southwestern margin of the species range. Analyses of seven microsatellite loci revealed 86 genets among 522 samples. In general, the phalanx strategy dominated in the populations considered, as 76% of ramets shared the same genotype with their closest neighbour. Nevertheless, substantial clonal and genetic diversities and high contribution of unrelated individuals in all *B. humilis* stands suggest that panmictic pollination prevails. On the other hand, positive and significant relationships between genetic and geographic distances in the two populations could be a consequence of biparental inbreeding resulting from the pollen and seed flow limitations. The seed germination capacity was very low (2.70%); however, the populations characterised by the lowest and highest values of clonal diversity parameters did not differ significantly in the number of germinated seeds, which indicates that clonality is not responsible for seed production failure.

Keywords Biparental inbreeding · Clonal propagation · Endangered species · Genetic relatedness · Microsatellites · Seeds germination

Introduction

Genetic differences between individuals are crucial for the adaptation and evolution of populations. As new gene combinations are generated by recombination processes, mating between unrelated (i.e. genetically different) individuals (outbreeding) increases genetic diversity. In turn, a substantial contribution of selfing or mating between close relatives (i.e. biparental inbreeding; Uyenoyama 1986) decreases the level of genetic variation. Compared to outcrossed individuals, inbred progeny can suffer from a fitness decline resulting from the accumulation of deleterious alleles (inbreeding depression; Glémin et al. 2001). As inbreeding is a consequence of a finite population size, small isolated populations of rare plant species seem to be especially threatened, as most individuals in such populations can represent a common ancestry (Frankham 1995). Most likely, the increased level of inbreeding in the two highly isolated Polish populations of English yew *Taxus baccata* L. was an effect of spatially restricted pollen flow and kinship structure (Chybicki et al. 2011). Spatial restriction of gene flow can be extorted or strengthened by a high density of individuals in the population. It was shown that pollen movement increased from 200 m in the high-density to 1000 m in the low-density populations of the timber tree *Erythrophleum suaveolens* (Guill. & Perr.) Brenan in central Africa (Duminil et al. 2016). *Taxus baccata* exemplifies the fact that even populations of wind-pollinated species can be structured due to limitations in pollen and seed dispersal (Chybicki and Oleksa 2018). Effective pollination by near neighbours was also demonstrated in other wind-pollinated species, such as southern beech *Nothofagus nervosa* (Phil.) Dim. et Mil. (Marchelli et al. 2012) and white oak *Quercus alba* L. (Smouse et al. 2001).

Limited gene exchange can be a conspicuous problem in clonally reproducing trees and shrubs that form less or more dense clusters of ramets that are genetically identical to

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parental organisms (genets) (Dering et al. 2015; García Cruzatty et al. 2017). Although long-lived clone branches can gain and accumulate somatic mutations during a lifetime, relatedness between parental and mutated ramets is still very high (James and McDougall 2014; Jankowska-Wróblewska et al. 2016). Clonal growth can enhance the rate of geitonogamy, i.e. pollination between flowers of the same plant (Harder and Barrett 1996), and consequently lead to inbreeding depression in self-compatible plants or decreased reproductive success and genetic diversity in self-incompatible species (Vekemans et al. 1998; Eckert 2000; Honnay and Jacquemyn 2008). The frequency of self-pollination should increase with clone size, as a higher number of ramets of the same clone increases the probability that two random flowers from a population belong to the same genet (Eckert 2000; Barrett 2015). However, in clonal plants, the rate of selfing or cross-fertilisation depends on the clonal architecture, i.e. the degree of intermingling of ramets from the same clone. Two growth strategies are characteristic for clonal plants: guerrilla and phalanx (Lovett-Doust 1981; Barrett 2015). In the guerrilla strategy, due to long distances between vegetative ramets, the inter-genet distances decrease and the clones are dispersed and intermixed, which facilitates cross-fertilisation. In contrast, the phalanx species have the vegetative ramets of one genet very close to the parental shoot. Thus, the mixing of ramets of different clones is significantly limited, making self-fertilisation more likely. These are two extreme types of clonal growth, but in reality, there is a continuum of the degree of clone mixing between these contrasting strategies (Charpentier 2002; Barrett 2015). Since the guerrilla strategy enables rapid spreading, it has an advantage during the succession and occupation of available space or in heterogeneous habitats, where it allows escape from unfavourable patches, while the phalanx type of growth occurs more often in habitats rich in resources or at high-density sites, increasing its competitive strength (Lovett-Doust 1981; Schmid and Harper 1985; Winkler and Schmid 1995). In homogenous habitats, under resource-rich conditions and open access to sunlight, aggregated growth of ramets allows them to remain at favourable sites, as it was shown in some clonal shrub species, such as *Rubia peregrina* L. (Navas and Garnier 2002) and *Robinia pseudoacacia* L. (Zhang et al. 2006).

Until now, little is known about the clonal structure and its potential influence on gene flow in the populations of the shrub birch *Betula humilis* Schrk. The shrub birch is a multi-branched, monoecious, wind-pollinated and wind-dispersed species that also reproduces vegetatively. Like other birches, the species is likely to be self-incompatible. The continuous range of *B. humilis* extends from central Europe to Siberia and northern Mongolia (Ashburner and McAllister 2016), but the plant is recognised as a glacial relict and listed as an endangered (EN category of the IUCN) species in central and western European countries (Calko 2014; Załuski et al. 2014). As other birches, *B. humilis* is photophilous plant; thus, its shading by brushwood and forest canopy, being a consequence of secondary succession at the sites with low groundwater levels, was recognised as a main cause of the species decline (Pogorzelec and Wojciechowska 2011; Jabłońska 2012; Załuski et al. 2014). Nuclear microsatellite analyses conducted in the randomly collected samples in the Polish marginal and Belarusian sub-central populations of *B. humilis* revealed a substantial level of genetic variation (Jadwiszczak et al. 2011a, b). The cladistic approach of matrix incompatibility strongly suggested that intra-population genotypic variation of the shrub birch resulted from frequent recombination events, i.e. effective sexual reproduction (Chrzanowska et al. 2016). On the other hand, an onset of genetic erosion was noted in the smallest and most isolated localities (Jadwiszczak et al. 2011a, b). Five out of 16 marginal populations were characterised by statistically significant values of the inbreeding coefficient (F_{IS}). Lowered germination ability of seeds in the Polish stands of the shrub birch compared to the Belarusian localities was also noted (Chrzanowska et al. 2016). Molecular studies conducted in the shrub birch population located in the Wizna mire, one of the biggest declining fens in Poland, located in northeastern part of the country, showed that the species might propagate only clonally in unfavourable habitats (Chrzanowska and Jadwiszczak 2015). As a significant decrease in the population numbers was noted in Poland during the twentieth century (Załuski et al. 2014), it is very urgent to assess the extent and potential meaning of clonal reproduction in *B. humilis*. We specifically addressed the following three questions: (1) Does the type of clonal growth depend on the light conditions? (2) Is the gene exchange spatially restricted in the populations studied? (3) Is seed germination more effective in more clonally diverse populations compared to less differentiated populations?

Materials and methods

Sampling sites

The studies were conducted in six *B. humilis* populations located in northeastern Poland: Sołtysek (SOL), Jeziorko (JEZ), Rospuda (ROS), Magdzie Bagno (MB), Góra Perkuć (GP) and Szuszalewo (SUS; Table 1). The degree of shading was estimated according to Chrzanowska et al. (2016) as 0, no shade (no canopy cover); 1, half shade (canopy cover of 50%) and 2, full shade (canopy cover of 100%) in each population. All populations occupy an area covered by the ice sheet during the last glaciation (see Jadwiszczak et al. 2011a); thus, they were established after the ice retreat and may be of a similar age. With the exception of the SUS, which is one of the shrub birch populations in the Biebrza National Park, the remaining localities are isolated and include a limited

Table 1 Names of *B. humilis* populations studied, their geographical coordinates and genetic and clonal diversity measures. Shade: 0 no shade, 1 half shade, 2 full shade; *N* number of sampled ramets, *MLL* number of multilocus lineages, *Ac* aggregation index, *A* mean number of microsatellite alleles per locus, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} inbreeding coefficient, *C* clonal richness, *E* clonal evenness, *D* Simpson's diversity index

	Population name	Population code	Coordinates		Shade	*N*	MLL	*Ac*	Genetic diversity				Clonal diversity		
			Latitude	Longitude					*A*	H_E	H_O	F_{IS}	*C*	*E*	*D*
1	Sołtysek reserve	SOL	53° 36′ 08″ N	20° 50′ 40″ E	2	73	9	0.852*	6.14	0.732	0.683	0.126	0.111	0.903	0.834
2	Jeziorko koło Drozdowa reserve	JEZ	53° 50′ 36″ N	21° 48′ 48″ E	0	89	18	0.763*	10.57	0.806	0.798	0.039	0.193	0.908	0.899
3	Rospuda valley	ROS	53° 54′ 23″ N	22° 56′ 38″ E	1	90	13	0.679*	8.86	0.807	0.879	−0.050	0.135	0.900	0.865
4	Magdzie Bagno swamp	MB	54° 08′ 41″ N	23° 16′ 05″ E	1	93	18	0.594*	8.57	0.798	0.873	−0.070	0.185	0.872	0.875
5	Góra Perkuć reserve	GP	53° 54′ 02″ N	23° 18′ 36″ E	1	85	19	0.722*	9.00	0.735	0.762	−0.024	0.214	0.950	0.930
6	Biebrza National Park, Szuszalewo	SUS	53° 43′ 07″ N	23° 21′ 23″ E	0	92	9	0.947*	8.29	0.814	0.746	0.141*	0.088	0.884	0.814
								Mean:	8.571	0.782	0.790	0.027	0.154	0.903	0.870

* Value statistically significant after the Bonferroni correction

area. The shrub birch branches are most numerous in SUS, followed by the ROS (these localities occupy the largest areas at the same time), while the SOL population is the smallest. The SOL locality is spread on a strongly degraded fen, while the remaining stands form shrublands on brown moss-small sedge sub-neutral fens. In the brown moss-small sedge fens, *Tomantypnum nitens* (Hedw.) Loeske, *Helodium blandowii* (Web. et Mohr.) Warnst., *Aulacomnium palustre* (Hedw.) Schwägr., *Plagiomnum ellipticum* (Brid.) T. Kop., and some *Sphagnum* species dominate in the moss layer; *Carex diandra* Schrk., *C. rostrata* Stokes, *Festuca rubra* L., *Comarum palustre* L. and *Menyanthes trifoliata* L. are found in the herb layer (Jabłońska 2012). Understory and canopy layers are mainly formed by: *Salix rosmarinifolia* (L.) Hartm., *S. cinerea* L., *Betula pubescens* Ehrh., *Alnus glutinosa* Gaertn. and *Frangula alnus* Mill. (Jabłońska 2009). As *B. humilis* prefers a groundwater table near the peat surface, fen degradation resulting from the water deficit is a threat for this weakly competitive species (Jabłońska 2012). Indeed, shrub birch forms few isolated groups of branches in the SOL, as its growth is strongly limited by willows *Salix* sp., nettles *Urtica dioica* L. and reeds *Phragmites australis* (Cav.)Trin. ex Steud. High groundwater levels are noted every year in the JEZ. Although still beneficial for *B. humilis*, the abundance of water has decreased in recent years in the ROS, MB and GP compared to that noted before 2009 (Jabłońska 2009). Lowering groundwater levels are responsible for the growth of competitive brushwood and forest species that have started to shade *B. humilis* in these populations. In the fully shaded SOL and half shaded MB, ROS and GP localities, *B. humilis* is tall (1–1.5 m), and in the JEZ and SUS, adult bushes are rather short (0.5–1 m). Few years ago, downy birch *B. pubescens* was removed from the SUS fen to preserve the endangered shrub birch. Now, the surroundings of this population are mowed every year.

Sampling

To study clonal architecture, four square plots (1.5 m × 1.5 m) were selected in each population, and the distribution of all ramets was mapped within each plot. With the exception of the SOL, where adult ramets were found only, ramets within each plot represented different age classes (young and older). As branches are unevenly distributed within the populations, the distance between plots in a single population depended on the location of similarly abundant clumps and ranged from 12 to 174 m. To conduct genetic analyses, two young leaves from each ramet were collected and preserved in plastic bags with silica gel. The leaves were transferred to the laboratory and stored at room temperature until DNA extraction. The total number of sampled ramets was 522. In the autumn, seeds of 30 individuals of each population were collected to test the germination rate. Collection of plant material was conducted according to permission nos. DOP-WPN.286.122.2017.RS, WOPN.6400.51.2017.PK, WPN.6400.33.2017 and WPN.6205.23.2018.MC.

Molecular analyses

Before DNA extraction, leaves were homogenised with the TissueLyser mill (Qiagen). Total genomic DNA was extracted from leaf material using an AX Plant Kit (A&A Biotechnology) according to the manufacturer's instructions. Genotyping of ramets was carried out using seven nuclear simple sequence repeats (SSRs) described for *Betula pendula* Roth (L1.10, L5.1, L5.4, L022; Kulju et al. 2004) and *Betula pubescens* Ehrh. (L021, Bo.F394, Bo.G182; Truong et al. 2005). Loci were chosen based on their significant variation that was revealed in the previous investigation conducted in the Polish and Belarusian populations of *B. humilis* (Jadwiszczak et al. 2011a). The usefulness of the loci was

checked previously by Jadwiszczak et al. (2011a) in MicroChecker 2.2.3 (van Oosterhout et al. 2004) by testing for stuttering, large allele drop-out and null alleles, and no potential genotyping errors were found. The primers were fluorescently marked and combined into three PCR multiplexes with different numbers of cycles: L1.10, L021 and L022 (24 cycles); L5.4 and L5.1 (27 cycles) and Bo.F394 and Bo.G182 (32 cycles). The proportions of the PCR components and the PCR profiles were the same as previously described by Jadwiszczak et al. (2011a). Amplification of microsatellites was carried out in a SensoQuest thermocycler (Biomedizinische Electronik). The separation of amplified fragments was conducted on an ABI PRISM 3130 sequencer (Applied Biosystems) with Gene Scan-500 LIZ size standard (Applied Biosystems) and scored using GeneMapper 4.0 software (Applied Biosystems).

Germination experiment

Before the experiment, 100 seeds from each individual were counted and stored at low temperatures to conduct vernalisation. Seeds were kept at + 4 °C from mid-December to mid-January, at − 20 °C from mid-January to 10th February and at 4 °C from 10th February to mid-April. Afterwards, seeds were placed in Petri dishes with filter paper and distilled water and placed in a phytotron at a constant temperature of 20 °C with a photoperiod of 10 h of light and 14 h of dark (Holm 1994). Every second day, germinated seeds were counted and removed. The germination experiment lasted 8 weeks and finished after no seeds germinated for 5 days (Holm 1994).

Data analyses

Determination of the number of different multilocus genotypes (MLGs) and assignment of samples to a particular MLG was performed with the use of GenClone 2.0 (Arnaud-Haond and Belkhir 2007). Genotyping was verified according to Arnaud-Haond et al. (2007a) to exclude two possible errors. The first involved finding identical MLGs arising from different zygotes, which can be caused by high genetic similarity and insufficient discriminative power of the genetic markers. The second was based on incorrect assignment of samples of the same clone to different MLGs, on account of somatic mutations or potential scoring errors (Halkett et al. 2005). To assess whether all replicates of the same MLG belong to the same clone, the probability that the repeated genotypes originated from distinct sexual reproductive events (p_{sex}) was calculated in GenClone. The Monte Carlo procedure was also applied to define a sufficient number of loci that provided enough power to discriminate all MLGs presented in the sample. To recognise distinct MLGs that could belong to the same clone or clonal lineage (MLL), the two-step approach proposed by Arnaud-Haond et al. (2007b) was carried out. A matrix of genetic distances was created in GenClone, and pairs of MLGs with the lowest distances were checked to find pairs distinct for only one or two loci. Afterwards, p_{sex} was re-estimated after the removal of distinct loci. When the probability was lower than 0.01, the slight differences between MLGs were considered to be a result of somatic mutations or scoring errors. Thus, those MLGs were treated as belonging to the same genet.

The following parameters of genetic diversity were calculated using GenAlEx 6.5 (Peakall and Smouse 2006): mean number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E). The individual inbreeding coefficient (F_{IS}) was estimated using FSTAT 2.9.3 (Goudet 1995). Significant departures from zero for F_{IS} values were tested through 1000 random permutations with the application of a sequential Bonferroni correction (Rice 1989). All of these calculations were performed at the genet level that is, including only one individual from the particular MLL.

Clonal diversity was assessed using three indices for each population: (1) clonal richness (C), which is the ratio of the genets number to the number of sampled ramets, $C = (G\text{-}1)/(N\text{-}1)$ (Dorken and Eckert 2001); (2) clonal evenness (E), which describes the equitability of the distribution of ramets among genets and (3) Simpson's diversity index (D; Pielou 1969), which is the measure of clonal heterogeneity and is influenced by both the richness and the relative abundance of different genets. All these indices were calculated using GenClone (Arnaud-Haond and Belkhir 2007). The spatial arrangement of genets was estimated based on the aggregation index Ac calculated in GenClone for each plot and each population. Its significance was determined by running 1000 permutations. To compare Ac parameters between plots with different degrees of shade (full shade, half shade, lack of shade), the Kruskal-Wallis ANOVA was performed with IBM SPSS Statistics 23 (George and Mallery 2016).

To visualise the genetic relationship between genotypes, a principal coordinates analysis (PCoA) was carried out using GenAlEx (Peakall and Smouse 2006). Two coordinates explaining the largest percentage of variation were plotted. Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed at both ramet and genet levels in Arlequin version 3.11 (Excoffier and Lischer 2010) to estimate genetic variation at three levels: among populations, among plots within populations and within plots. The significance of genetic variation was estimated using 1000 permutations.

Maximum likelihood estimates of relatedness (R) for pairs of genotypes within each locality were estimated in ML-RELATE (Kalinowski et al. 2006); then, the values were averaged for a population. Genotype pairs were classified as unrelated (U; $R = 0$), half-siblings (HS; $R = 0.25$), full-siblings (FS; $R = 0.5$) and parent-offspring (PO; $R = 0.5$). Testing of the consistency of relationships with the genetic

data was carried out at the 95% confidence level (Kalinowski et al. 2006) with 1000 permutations. To determine whether genetic distances between pairs of genets were correlated with their geographical distribution, a Mantel test (Mantel 1967) was performed for each population using Alleles In Space (Miller 2005). The geographical position of the central ramet was taken into consideration, and in the absence of such ramet, the central position was interpolated. The statistical significance of the correlations was tested by running 1000 permutations.

To compare the germination ability of seeds among populations, the Kruskal-Wallis ANOVA was conducted in IBM SPSS Statistics 23 with a post hoc test.

Results

Permutations in GenClone showed that just four loci would allow the identification of all distinct MLGs. The p_{sex} of all the samples was lower than 0.01; thus, it can be assumed that identical MLGs were derived from the same clone. After screening the pairs of MLGs with the lowest genetic distances and recalculating p_{sex}, one pair of MLGs from the GP population turned out to belong to the same MLL. All samples sharing the same MLL were considered to belong to the same genet in all subsequent analyses. In total, 86 MLLs were revealed, of which 21 were sampled only once. There was a range of one to nine genets in a single plot and nine to 19 in a single population (Table 1, Fig. 1). In most plots, few genets were found, although in all populations, plots containing only one or two clones were noted. No plots shared the same MLL in any population.

All loci studied were polymorphic. The average number of alleles ranged from 6.14 in the SOL population to 10.57 in the JEZ population (Table 1). Observed and expected heterozygosities across all populations were high, with mean values of 0.790 and 0.782, respectively. Estimates of the inbreeding coefficient showed a significant excess of homozygotes in the SUS population only ($F_{IS} = 0.141$, $P = 0.005$). Clonal richness and Simpson's diversity indices were the lowest in the SUS population ($C = 0.088$ and $D = 0.814$, respectively) and the highest in the GP population (0.214 and 0.93, respectively; Table 1). Clonal evenness (E) ranged from 0.872 in MB to 0.95 in GP.

The aggregation index (Ac) varied from 0.594 in MB to 0.947 in SUS, with an average of 0.76, which indicated that 76% of ramets shared the same genotype with their closest neighbour (Table 1). This means that ramets of the same genet were rather closely aggregated. All values of Ac were statistically significant ($P < 0.0001$). There were no statistically significant differences in Ac between plots with different access to sunlight ($H = 2.086$, $P = 0.352$). In the PCoA, the first and the second axes explained 8.17% and 6.47% of the total variance, respectively (Fig. 2). Some of the MLLs derived from the same plot were grouped together in the PCoA, e.g. genotypes from plot 14 (MB locality) and 19 (GP), but most of the MLLs were intermixed. The hierarchical AMOVA showed that most of the genetic variation was found within plots of both analyses at the ramet (63.66%, $F_{ST} = 0.363$, $P < 0.0001$) and genet (89.23%, $F_{ST} = 0.108$, $P < 0.0001$; Table 2) levels, while the variation between populations was low, albeit significant.

Values of the mean relatedness estimator ranged from $R = 0.0236$ in the SUS population to 0.0669 in the MB locality, which reflected the highest (94.4%) and lowest (81.7%) contributions of unrelated individuals (U) in these populations, respectively. Half-siblings (HS) were noted in all populations studied, but they were the most frequent in MB (13.1%). There were no full-siblings (FS) in the SOL and SUS populations, and this category was the rarest in the remaining localities. Parent-offspring (PO) pairs were not revealed in SUS, and their contribution was the highest in ROS (5.1%). Positive and significant correlations of genetic and geographic distances were detected in ROS and GP (Table 3).

In the germination experiment, only 2.70% of seeds sprouted. Germination capacity (*GS*; median number of germinated seeds) was 3.5 in the SOL, 2 in MB, 0.5 in SUS and 0 in the remaining populations. The distribution of germinated seeds is presented in Fig. 3. The Kruskal-Wallis test revealed statistically significant differences in germination capacity between the following pairs of populations: GP and SOL ($P_{adj} = 0.000$), GP and MB ($P_{adj} = 0.000$), JEZ and SOL ($P_{adj} = 0.000$), JEZ and MB ($P_{adj} = 0.000$) and ROS and SOL ($P_{adj} = 0.003$), as well as ROS and MB ($P_{adj} = 0.007$).

Discussion

In the present study, nuclear SSR markers were used to describe the clonal propagation pattern and its potential influence on gene flow and seed production in the endangered populations of *B. humilis*. Among 522 ramets, 87 different multilocus genotypes (MLGs) were found. The mean clonal richness ($C = 0.156$) of the shrub birch was considerably lower compared to the ratio of *G/N* which equalled 0.44 for clonal plants (Honnay and Jacquemyn 2008). A high value of *G/N* can be explained by two factors. The first is the overdominance of clonal reproduction in the shrub birch. This seems to be likely because, along with permanent seed banks and an extended life span, clonal propagation was described as a mode of survival for those plant species that were not able to reproduce sexually due to unfavourable habitat conditions (Alsos et al. 2002; García and Zamora 2003). In highly shaded by *B. pubescens* and *Salix cinerea* L. population of shrub birch located in the Wizna mire in northeastern Poland, three multistem clones were found only, suggesting sole vegetative

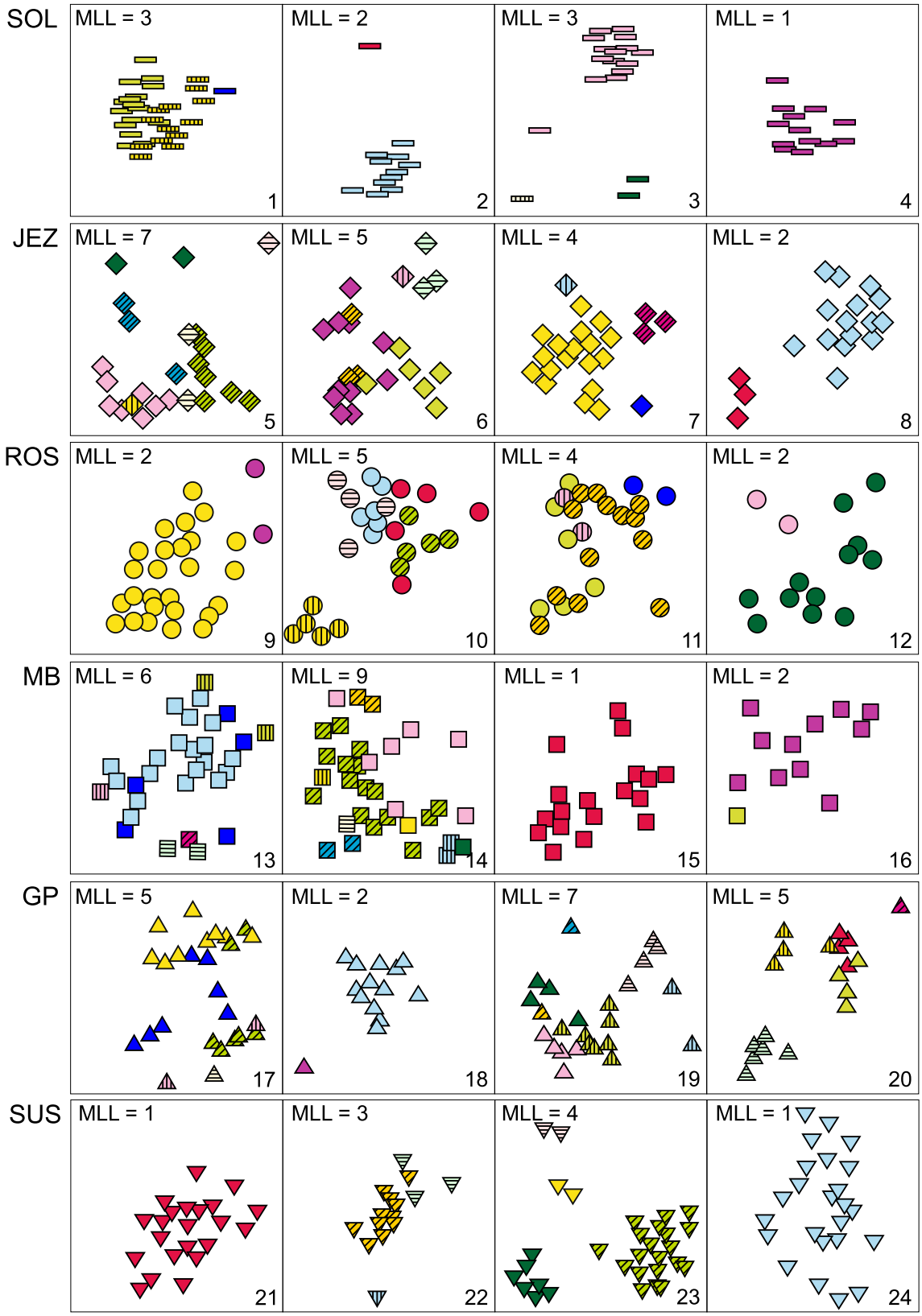

Fig. 1 Spatial distribution of *B. humilis* clones within studied plots (1.5 × 1.5 m). Different shape symbols are designed for particular populations. Ramets belonging to the same multilocus lineage (MLL) are marked with the same colour symbol. Numbers of the plots are given in the bottom right corner of each plot. Population codes according to Table 1

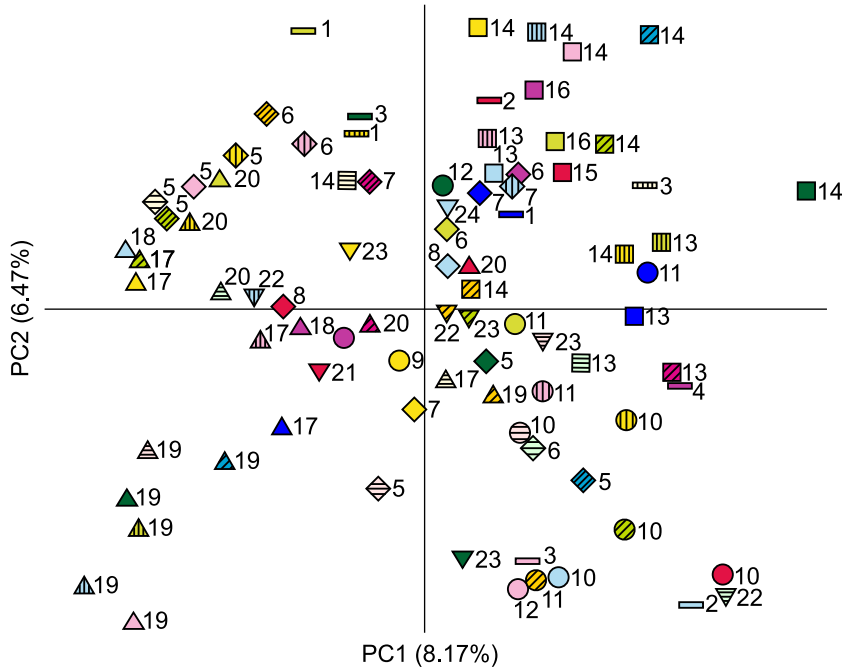

Fig. 2 Principal coordinates analysis (PCoA) representing genetic distances between *B. humilis* multilocus lineages (MLLs). Numbers of plots and symbols of MLLs are the same as in the Fig. 1

propagation in that place (Chrzanowska and Jadwiszczak 2015). In the present study, the lowest *C* values were noted in the SUS (0.088) population, which experienced shading recently, and in SOL (0.111), which is now entirely overgrown by competitive plants. It has not been excluded, however, that low *C* values in the *B. humilis* localities may have resulted from the sampling strategy. The *C* parameter is heavily influenced by sample size and sampling scheme; thus, it should be interpreted with caution (Gitzendanner et al. 2012). The *G/N* ratio can be significantly lower when dense clumps are sampled because it increases the probability of collecting few samples of the same genet; when ramets are evenly distributed, the *G/N* ratio is higher. Allozyme analyses revealed that an average of 97% of trees sampled in six California populations of the oak *Quercus chrysolepis* Liebm. represented different genotypes, but clustered trees usually constituted single clones (Montalvo et al. 1997).

Ten out of 24 sample plots established in the shrub birch localities included only one or two MLLs. The distribution and number of different genotypes within sampling plots in the *B. humilis* populations strongly suggest that the phalanx strategy of clonal growth predominates. This means that derivative ramets are very close to paternal shoots and that different clones mix with one another to a low extent (Lovett-Doust 1981; Barrett 2015). This aggregation tendency was confirmed by high values of the aggregation indices (*Ac*) ranging from 0.594 to 0.947. This parameter was the highest in the SUS (0.947) and SOL (0.852) populations. It seems likely that the presence of competitive plant species in the SOL population makes the spread of shrub birch clones difficult. The SOL reserve covers an area of degraded peat bog surrounded by swamp forest. The *B. humilis* population is very small, and bushes are divided into few groups separated by dense reeds. High competition for light and space can limit seedling

Table 2 Analysis of molecular variance (AMOVA) for the *B. humilis* populations at the ramet and genet level

Source of variation	d.f.	Variance	% of variation	Fixation indices
Ramet level				
Among populations	5	0.13	4.41	0.044*
Among plots within populations	18	0.98	31.93	0.334*
Within plots	1020	1.95	63.66	0.363*
Genet level				
Among populations	5	0.15	5.83	0.058*
Among plots within populations	18	0.13	4.94	0.052*
Within plots	156	2.33	89.23	0.108*

* Values statistically significant, $P < 0.0001$

Table 3 Mean value of relatedness estimator (*R*), contributions of unrelated individuals (U), half-siblings (HS), full-siblings (FS) and parent offspring specimens (PO) and results of the Mantel tests (*r*) comparing genetic and geographic distance matrices in the *B. humilis* populations. Population codes according to Table 1

Population	*R*	Contribution (%) of				Mantel test
		U	HS	FS	PO	*r*
SOL	0.0375	91.7	5.6	0	2.8	0.057
JEZ	0.0433	92.2	3.9	0.7	3.3	0.245
ROS	0.0576	88.5	5.1	1.3	5.1	0.387*
MB	0.0669	81.7	13.1	1.3	3.9	0.060
GP	0.0577	87.9	8.4	1.6	2.1	0.330*
SUS	0.0236	94.4	5.6	0	0	0.357

*Values statistically significant after the Bonferroni correction

recruitment and divert the allocation of resources into vegetative growth. For example, *Uvularia perfoliata* L., a temperate deciduous woodland perennial, formed a large number of genets in canopy gap habitats, while at the closed canopy sites, patches consisted of a single genet with no flowering shoots (Kudoh et al. 1999). We think that without urgent conservation practices, *B. humilis* from the SOL can share the fate of plants from the Wizna mire (Chrzanowska and Jadwiszczak 2015) in the near future. In our opinion, the reasons for the considerable aggregation of *B. humilis* in the SUS can be quite different. Few years ago, shrub birch bushes declined in this area due to the overgrowth of other plants. After removing *B. pubescens* and other competitive species, a newly freed space allowed for rapid vegetative propagation of *B. humilis*. The study by Sammul et al. (2004), comparing plant communities in different habitats, showed a positive effect of mowing on ramet density at open sites and revealed that in open meadows, the clonal mobility of plants was lower and branching was more intense compared with brushwood or forest sites, where light was distributed heterogeneously. In fact, most of the shrub birch populations fitted into this scheme. However, this pattern was not observed in the SOL, which was the most shaded population, but the shrub birch clones were highly aggregated. Thus, no significant differences in clumps aggregation were observed among plots subjected to different shade conditions.

The highest numbers of distinct MLLs were counted in the *B. humilis* populations situated in undisturbed well saturated fens: JEZ, ROS, GP and MB. In those localities, a differentiated pattern of clonal growth was observed. Some sampling plots were dominated by single genotypes, but other patches comprised up to nine distinct genets. Shrub birch ramets showed the lowest aggregation in the MB ($Ac = 0.594$) and could exhibit an intermediate growth strategy between phalanx and guerrilla at this site. Based on the morphology of underground parts of few individuals in the Narew River valley (northeastern Poland), Szańkowski (1991) found that the species could form both clumped and dispersed bushes. Plasticity of clonal growth was described in the dwarf shrub *Rhododendron aureum* Georgi as an adaptive mechanism

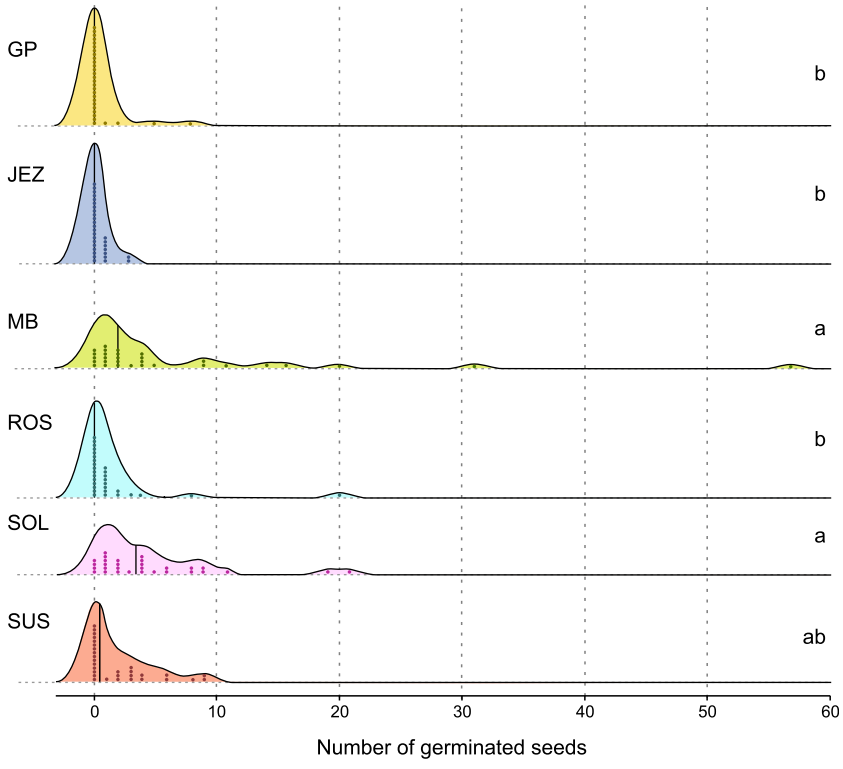

Fig. 3 Distributions of germinated seeds in the *B. humilis* populations ordered according to the increasing value of Simpson's diversity index (*D*; Table 1). Each dot indicates a number of germinated seeds of a single individual. Vertical continuous lines show medians of populations. Different letters indicate significant differences between populations (Kruskal-Wallis ANOVA; $P < 0.05$). Population codes according to Table 1

allowing for this plant to colonise and exploit tundra and birch forest in Changbai Mountain in China (Wang et al. 2018). In general, the predominance of some *B. humilis* clones can imply their selective advantage over other genets; however, there is still a substantial overall clonal diversity in all populations analysed. Clonal evenness ranged from $E = 0.872$ to 0.950, with a mean of 0.902, and Simpson's diversity index (D) ranged from 0.814 to 0.930 (mean value of $D = 0.870$). These values were higher than both of these parameters in the multiclonal plants (0.68 and 0.62, respectively; Ellstrand and Roose 1987) as well as in self-incompatible species (0.67 and 0.75, respectively; Honnay and Jacquemyn 2008). High values of clonal diversity parameters in the shrub birch locations can result from frequent recombination events being a consequence of effective sexual reproduction. Using AFLP markers, it was shown that most randomly sampled shrub birch individuals originated from outcrossed matings (Chrzanowska et al. 2016).

Multiple lines of genetic evidence in this study also suggest that sexual reproduction of *B. humilis* can be effective. First, all populations included multiple genets belonging to different MLLs, most likely formed by recombination (see Ellstrand and Roose 1987). Two MLGs from only the GP population belonged to the same clonal lineage. In five localities of the wild service tree *Sorbus torminalis* (L.) Crantz situated in northern Poland, 42% of trees belonged to clonal groups (Jankowska-Wróblewska et al. 2016). Second, parameters of genetic diversity were surprisingly high. The mean values of the numbers of microsatellite alleles per locus (A), and observed (H_O) and expected (H_E) heterozygosities were 8.571, 0.782 and 0.79, respectively, and they were even higher than those described in previous microsatellite studies (Jadwiszczak et al. 2011a, b). One should remember, however, that highly variable SSR loci only were considered in the present analyses. Third, at both the ramet and genet levels, the hierarchical AMOVAs revealed that most of the genetic variation was found within the plots. Genetic differentiation between the shrub birch populations at both the ramet ($F_{ST} = 0.044$) and genet ($F_{ST} = 0.058$) levels was rather low but significant.

Considerable genetic variation excludes an increased selfing rate by geitonogamy in *B. humilis*, although it was suggested that the phalanx type of growth could significantly increase the selfing among ramets of the same genet, decreasing the chance for outcrossing at the same time (Handel 1985; Charpentier 2002; Barrett 2015). Albert et al. (2008) showed that the selfing rate in the woody perennial *Vaccinium myrtillus* L. was largely and significantly higher for plants in patches with a low number of genets than in patches characterised by a high number of more intermingled clones (50% and 3%, respectively). At present, we are not sure if self-fertilisation is totally absent in *B. humilis* because some birch species were shown to be self-fertile to some extent (Clausen 1966). Self-fertilisation experiments, which are planned in the future in shrub birch localities, should resolve this problem. It is expected that populations of partially asexual self-incompatible species should be characterised by slightly negative F_{IS} values (Stoeckel and Masson 2014), as was shown in the wild trees *Prunus avium* L. (Stoeckel et al. 2006) and *S. torminalis* (Jankowska-Wróblewska et al. 2016). A negative inbreeding coefficient was noted in ROS, MB and GP stands of *B. humilis*. A population genetics model has predicted that increasing rates of asexual reproduction should decrease the probability of observing positive F_{IS} (Stoeckel and Masson 2014), but this did not occur in the SUS ($F_{IS} = 0.141$), which seems to express the most intensive clonal propagation. Two explanations are possible. First, clonal propagation is not a main mode of reproduction in the whole locality. Second, the SUS sample can exemplify a specific distribution of F_{IS} values revealed in highly asexual populations. In such populations, F_{IS} value distributions strongly shifted to negative values but also spread the right tails into high positive F_{IS} values compared to fully sexual and intermediate asexual populations (Stoeckel and Masson 2014).

The selfing rate in *B. humilis* has not been studied directly until now, but our investigation revealed another interesting result. Some adjacent genets shared the same alleles and were grouped together in the PCoA ordination. This was especially clear for the MLLs from plot 19 in GP and to a lesser extent in plots 5 in JEZ and 14 in MB. Although unrelated (U) individuals clearly dominated in all populations analysed, the presence of genets sharing some alleles within a plot can imply local dispersion of pollen and seeds. In general, populations of wind-pollinated trees were recognised to be panmictic over large spatial scales (Ashley 2010); however, limitations in pollen flow were also described. The kinship structure detected in *T. baccata* populations was likely an effect of the fact that the majority of pollen grains fall on nearby trees. This phenomenon was clearer in denser stand than in less compacted tree group (Chybicki et al. 2011). Effective pollen dispersal also depended on the density of individuals in the *E. suaveolens* populations, being highest within the groups of most separated trees (Duminil et al. 2016). *Betula humilis* bushes are not high, and ramets can form dense clumps; thus, we expected that pollen flow could also be reduced in this species increasing the chance of biparental inbreeding. Indeed, significant relationships between genetic and geographical distance matrices in the GP and ROS seem to support this hypothesis. It is interesting why this phenomenon was not observed in MB, although it is overgrown and shaded similarly to ROS and GP populations. Moreover, the value of the relatedness parameter was the highest ($R = 0.0669$) in MB and was slightly lower in GP (0.0577) and ROS (0.0576). The highest R value in the MB locality is a consequence of the substantial contribution of half-siblings (HS = 13.1%), but the lack of a statistically significant r value of the Mantel test strongly suggests that related individuals are randomly distributed within this place. We suppose that a lack of relationship

between genetic and geographical distances in MB can result from even dispersion of pollen and seeds within a very small area occupied by shrub birch individuals.

A relatively high number of HS in the MB population can indicate that some genets are selectively advantageous. Indeed, more than half (52.2%) of germinated seeds in the MB came from three individuals only, which produced 57, 31 and 20 sprouting seeds in the germination experiment. Dominance of few genets can imply that remaining genotypes include the same incompatibility alleles which in consequence results in pollination limitation and seed production failure (Weis and Hermanutz 1993; Vekemans et al. 1998). In general, the total share of germinated seeds in the *B. humilis* experiment was very low (2.70%). It was suggested previously that a low number of sprouting seeds in the shrub birch populations depended on habitat conditions because seeds collected at the unshaded sites with high groundwater levels were heavier and more likely to sprout (Chrzanowska et al. 2016). Based on the present results, water abundance and shade do not seem to be the only factors affecting seed sprouting because germination capacity was the highest in SOL ($GS = 3.5$) and MB ($GS = 2$), being fully and half shaded, respectively. It was stated that clonal architecture and clonal diversity can significantly influence reproductive success (Vallejo-Marín et al. 2010; Barrett 2015; Van Drunen et al. 2015). Notwithstanding, both populations with the highest number of germinated seeds differed in terms of clonal growth. Most likely, despite the small population size and the relatively high contribution of close relatives, the low aggregation of clones in MB ($Ac = 0.594$) facilitates cross-fertilisation. On the other hand, as the SOL population represented a typical phalanx strategy, it is likely that clumped growth of shrub birch genets does not prevent successful pollen spreading. Seed production in the *B. humilis* populations seems not to be dependent also on the clonal diversity, as in the SUS and GP populations, characterised by the lowest and highest values of clonal diversity parameters, respectively, median values of GS were very similar. Further work is needed to identify factors responsible for the production of inviable seeds in the *B. humilis* populations.

Conclusions

This study revealed substantial clonal diversity of the shrub birch at the south-western margin of its range. Thus, genetic depauperation caused by excess clonal growth over sexual reproduction does not seem to be the main factor threatening *B. humilis* populations. The balance between these two reproduction strategies can increase the overall fitness of the individual instead of interfering with one other. According to Vallejo-Marín et al. (2010), the fitness of genets can potentially increase as a result of the production of numerous ramets with lower per ramet investment in reproduction. A recent study by van Drunen et al. (2015) showed that clonal plants could increase fitness through extended spatial expansion. Widespread clonal growth provides pollen dispersion over a larger area and a reduction in sibling competition for the area over which seeds can be spread. Despite both phalanx growth in the *B. humilis* populations and presumed small size of particular clones, self-pollination does not seem to be a considerable threat, even in small localities and overgrown sites, as unrelated individuals clearly predominate. It seems possible that the plasticity of clonal growth strategies and the benefits of clonal propagation, such as rapid growth and increasing competitive strength, can facilitate survival of the shrub birch and allow it to persist under different environmental conditions. Moreover, it is still likely that seed germination and seedling development could proceed in gaps among established genets, as was implied in another phalanx species, *Cirsium rivulare* (Jacq.) All. (Lembicz et al. 2011). However, the intense clonal growth seems to be insufficient for the shrub birch maintenance at the most overgrown sites. As lowering the groundwater levels leads to a significant decline in the size of the population, the smallest and the most overgrown stands require urgent active protection, as it was proposed by Jadwiszczak et al. (2012).

Data archiving statement Microsatellite genotype dataset is available on the Zenodo: https://doi.org/10.5281/zenodo.2477372.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Conclusions

In my PhD dissertation, I studied the impact of habitat conditions and different aspects of plant biology, such as sexual reproduction, hybridisation and clonal propagation, on the genetic variation of endangered marginal *B. humilis* populations. In accordance with previous studies, the genetic diversity of the shrub birch at AFLP loci was comparable among most peripheral and sub-central localities investigated (**Chapter II**). It is likely that genetic variation results from successful generative reproduction, as most genotypes resulted from meiotic recombination. However, I found a lower seed germination capacity in marginal stands than in the sub-central localities; thus, **Hypothesis 1** assuming no differences in reproductive performance across the *B. humilis* range was not confirmed (**Chapter II**). The reproduction efficiency of the shrub birch seems to be reduced under unfavourable habitat conditions, according to the assumption of **Hypothesis 2** (**Chapter II**). The germination capacity, which was positively correlated with seed mass, was significantly lower in dry stands than in wet habitats. Although a reduction in genetic variation resulting from scarce generative reproduction in some *B. humilis* populations was not observed, long-term disturbance of sexual reproduction efficiency could decrease genetic diversity in the future. Most likely, the observed substantial level of genetic variation in the shrub birch populations reflects the historic good condition of the populations, as the detected genotypes were formed in the past, while limited sexual reproduction can indicate a current weakened condition of individuals.

Declining shrub birch populations were expected to be extraordinarily vulnerable to crossbreeding with sympatric congeners. Due to the very low frequency of detected hybrids in the studied populations, the overall impact of hybridisation on the *B. humilis* gene pool seems to be minor; thus, **Hypothesis 3** was rejected. However, the three potential hybrids found in the *B. humilis* stands with low groundwater levels indicated that gene inflow from widespread tree birches could be a threat for the shrub birch in drained localities (**Chapter III**).

Unfavourable environmental conditions can impede or prevent sexual reproduction in plant populations; therefore, local persistence and increases in size can be provided by vegetative propagation (Barrett 2015). In fact, the analysis of the shrub birch population near Maliszewskie Lake indicated that in highly disturbed stands, the number of genetic individuals can be very low (**Chapter I**). An extreme reduction in population size caused a

drastic decrease in genetic variation; thus, natural restoration of a significant number of genetically diverse individuals became impossible. However, *B. humilis* was still able to persist in Wizna mire due to clonal reproduction.

Significant aggregation of clones can reduce gene exchange and impede outcrossing (Barrett 2015, Dering et al. 2015). The analysis of clonal architecture revealed that the shrub birches formed dense clumps of ramets belonging to the same clone (**Chapter IV**). In general, the aggregated growth of clones does not seem to hinder successful pollination between distinct genets, as substantial clonal and genetic diversities and a high contribution of unrelated individuals in all *B. humilis* populations were found (**Hypothesis 4**). However, pollen and seed dispersal can be limited to some extent in some endangered populations of the shrub birch. At Maliszewskie Lake, a single site was overgrown by ramets belonging to only one genet (**Chapter I**), which implies no seed exchange between ramet clumps. In turn, positive and significant relationships of genetic and geographical distances between pairs of genets in the Rospuda valley and Góra Perkuć reserve (**Chapter IV**) suggest biparental inbreeding. It is worth emphasizing that clonal growth alone does not appear to reduce the genetic diversity of *B. humilis*, as in most localities, generative reproduction occurs simultaneously. Instead, it allows persistence of the population under adverse conditions, and when habitat conditions are improved, clonal propagation is the main mode of individual spread.

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STATEMENT

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in the paper: **Chrzanowska A., Jadwiszczak K.A., Kłosowski S., Banaszek A., Sozinov O.V. 2016. Sexual reproduction efficiency and genetic diversity of endangered *Betula humilis* Schrk. populations from edge and sub-central parts of its range. Folia Geobotanica 51:161-173,** included co-authorship of the general concept of the work, collection of samples, reagents contribution, supervision of laboratory work, participation in data analysis and manuscript preparation;

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Chrzanowska A., Jadwiszczak K., Kłosowski S., Banaszek A., Sozinov O.V. 2016. Sexual reproduction efficiency and genetic diversity of endangered *Betula humilis* Schrk. populations from edge and sub-central parts of its range. Folia Geobotanica 51:161-173

included collection of samples, carrying out the chemical analyses of groundwater, advisory at manuscript preparation.

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Białystok, 29.05.2019

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STATEMENT

I declare that my contribution in the paper

Chrzanowska A., Jadwiszczak K., Kłosowski S., Banaszek A., Sozinov O.V. 2016. Sexual reproduction efficiency and genetic diversity of endangered *Betula humilis* Schrk. populations from edge and sub-central parts of its range. Folia Geobotanica 51:161-173,

included advisory in the manuscript preparation.

Agata Banaszek

Grodno, 10.05.2019

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STATEMENT

I declare that my contribution in the paper

Chrzanowska A., Jadwiszczak K., Kłosowski S., Banaszek A., Sozinov O.V. 2016. Sexual reproduction efficiency and genetic diversity of endangered *Betula humilis* Schrk. populations from edge and sub-central parts of its range. Folia Geobotanica 51:161-173,

included collection of leaves samples.

.....

Oleg V. Sozinov

Sofia, 13.05.19
place, date

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STATEMENT

I declare that my contribution in the paper

Bona A., Petrova G., Jadwiszczak K.A. 2018. Unfavourable habitat conditions can facilitate hybridisation between the endangered *Betula humilis* and its widespread relatives *B. pendula* and *B. pubescens*. Plant Ecology & Diversity 11:295-306

included participation in laboratory work and data analysis.

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Galya Petrova

Białystok, 5.06.2019

STATEMENT

I declare that my contribution in the paper

Bona A., Kulesza U., Jadwiszczak K.A. 2019. Clonal diversity, gene flow and seed production in endangered populations of *Betula humilis* Schrk. Tree Genetics & Genomes, DOI: 10.1007/s11295-019-1357-2

included participation in laboratory work.

Urszula Kulesza

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