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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

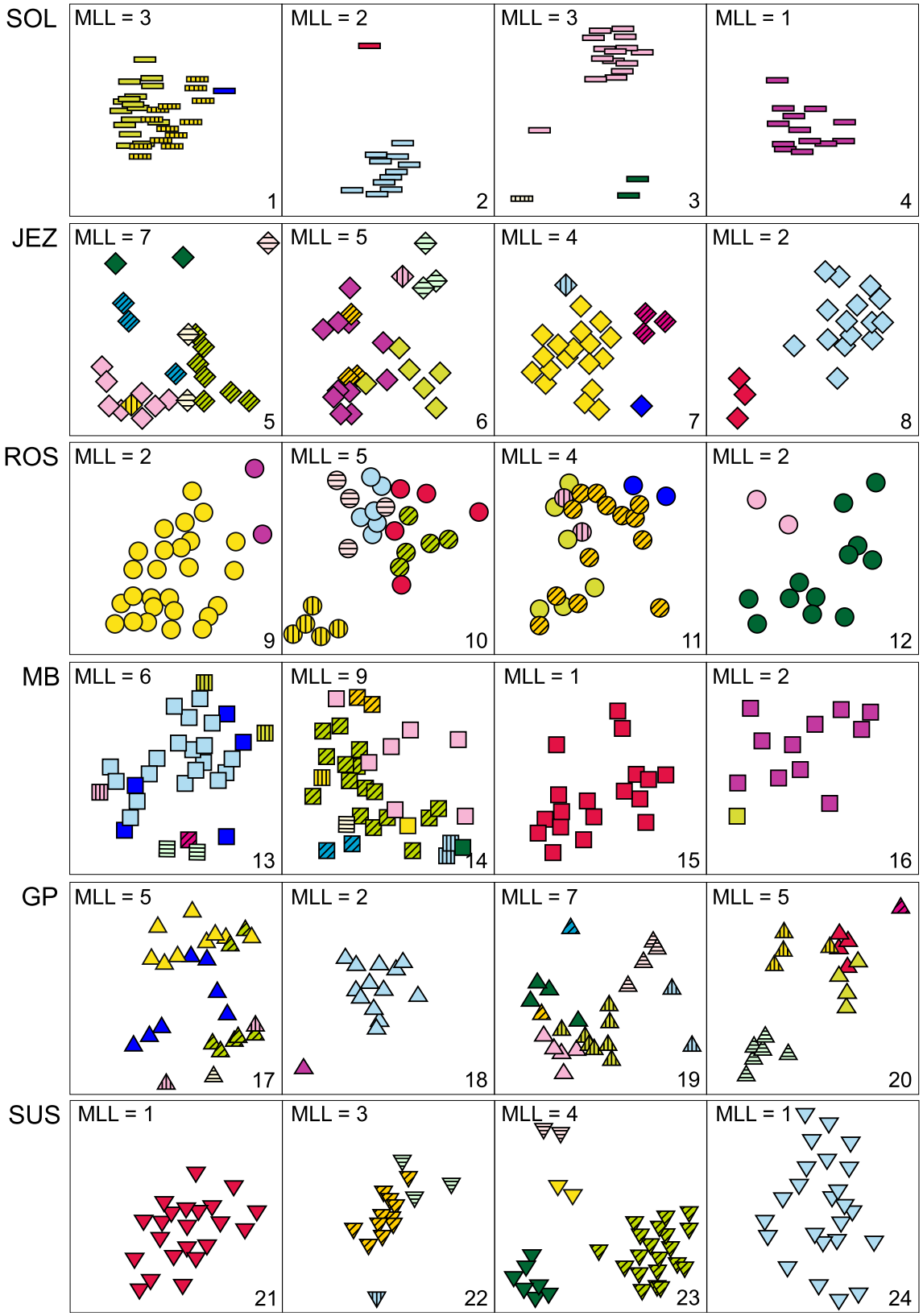


**Fig. 1** Spatial distribution of *B. humilis* clones within studied plots (1.5 × 1.5 m). Different shape symbols are designed for particular populations. Ramets belonging to the same multilocus lineage (MLL) are marked with the same colour symbol. Numbers of the plots are given in the bottom right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

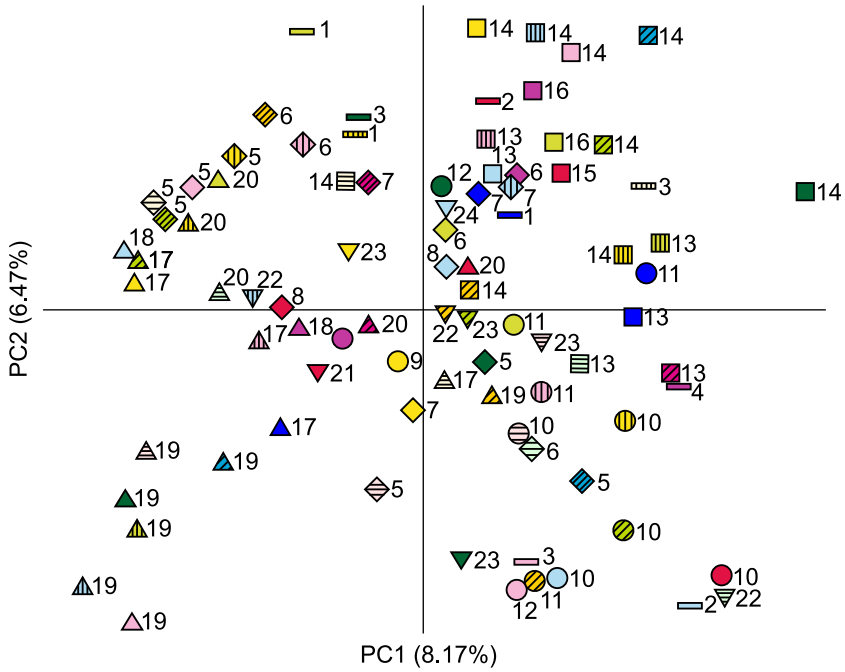


**Fig. 2** Principal coordinates analysis (PCoA) representing genetic distances between *B. humilis* multilocus lineages (MLLs). Numbers of plots and symbols of MLLs are the same as in the Fig. 1

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

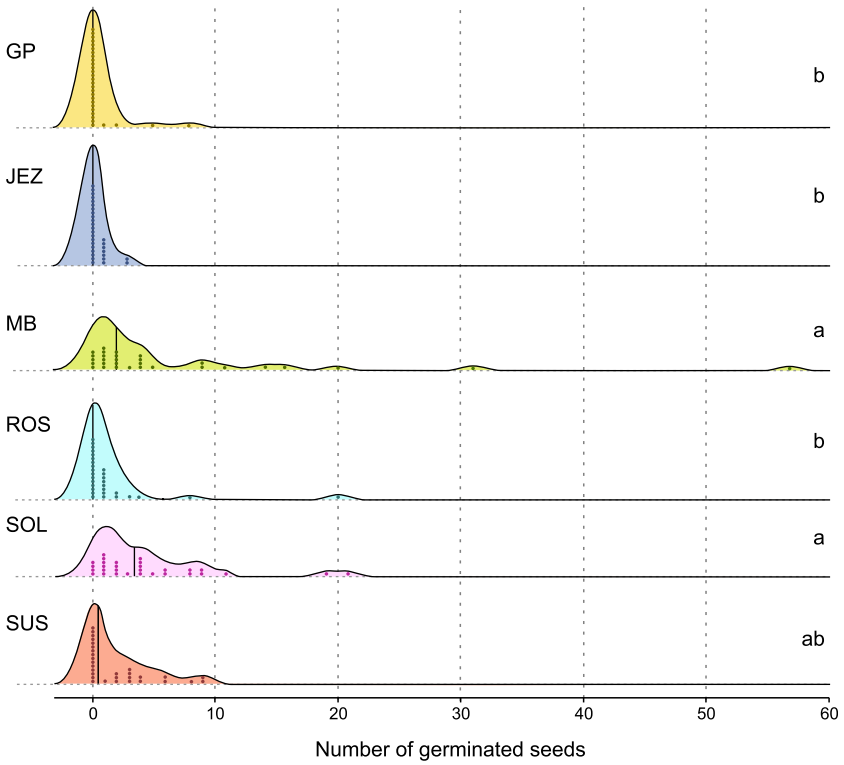


**Fig. 3** Distributions of germinated seeds in the *B. humilis* populations ordered according to the increasing value of Simpson's diversity index (*D*; Table 1). Each dot indicates a number of germinated seeds of a single individual. Vertical continuous lines show medians of populations. Different 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).

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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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