JOURNAL OF FOREST SCIENCE, *63*, 2017 (12): 531–537

doi: 10.17221/105/2017-JFS

Morphological variability between diploid and tetraploid taxa of the genus *Betula* L. in the Czech Republic

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Abstract

Linda R., Kuneš I., Baláš M., Gallo J. (2017): Morphological variability between diploid and tetraploid taxa of the genus *Betula* L. in the Czech Republic. J. For. Sci., 63: 531–537.

The aim of this work was to suggest a reliable method for distinguishing between diploid and tetraploid taxa of the genus *Betula* Linnaeus, based on leaf measurements. In total, 97 individuals from 6 locations within the Bohemian Forest region (Czech Republic) were selected. Four leaves from each individual were evaluated. On each leaf, twenty parameters were measured. Each sample was analysed by flow cytometry to determine its actual ploidy. Measured parameters were analysed by principal component analysis and tested for differences between diploid and tetraploid taxa. For actual ploidy prediction, a classification function was designed. The reliability of the classification function was verified on samples from three different regions of the Czech Republic and compared with functions as suggested by other authors. The classification function designed in this work (based on 3 parameters – blade width in the upper 1/4 of blade length, first vein angle and number of leaf teeth between 3rd and 4th vein) correctly determined actual ploidy in 89% of all tested samples.

Keywords: silver birch; downy birch; ploidy; flow cytometry; classification function

Taxonomic treatment within the genus *Betula* Linnaeus is generally considered problematic (Atkinson, Codling 1986; Howland et al. 1995; Grimm, Renner 2013). The uncertainties in the taxonomy of birches are mainly caused by massive hybridization and successive introgression (backcrossing with parental individuals) in the past (Howland et al. 1995; Palmé et al. 2004). Some of the birch taxa were likely given multiple names making the taxonomy of birches even more controversial (Wielgolaski 2001; Järvinen et al. 2004; Ashburner, McAllister 2013).

In the Czech Republic, there are two main naturally occurring birch species – silver birch (*Betula pendula* Roth) and downy birch (*Betula pubescens* Ehrhart) (Kříž 1990) that are important for forestry (Ministry of Agriculture of the Czech Republic Decree No. 84/1996). Both taxa naturally occur from lowlands to the mountain locations. Silver birch does not generally prosper above 1,000 m a.s.l., but in contrast to downy birch, it is almost evenly distributed across the Czech Republic. Downy birch occurs in relatively separated populations in wetlands or peat bogs, while its natural range could reach up to the highest altitudes of the Czech Republic.

Silver birch has a supportive role in local commercial forests (Kula 2011) due to its ameliorative properties (Zerbe 2001). Silver birch can be successfully used for substitute stands, for spoil tips or for the afforestation of agricultural lands at

Supported by the Czech University of Life Sciences Prague, Projects No. 20104308, No. 201130 and No. B03/17, and by the Ministry of Agriculture of the Czech Republic, Project No. QH 92087.

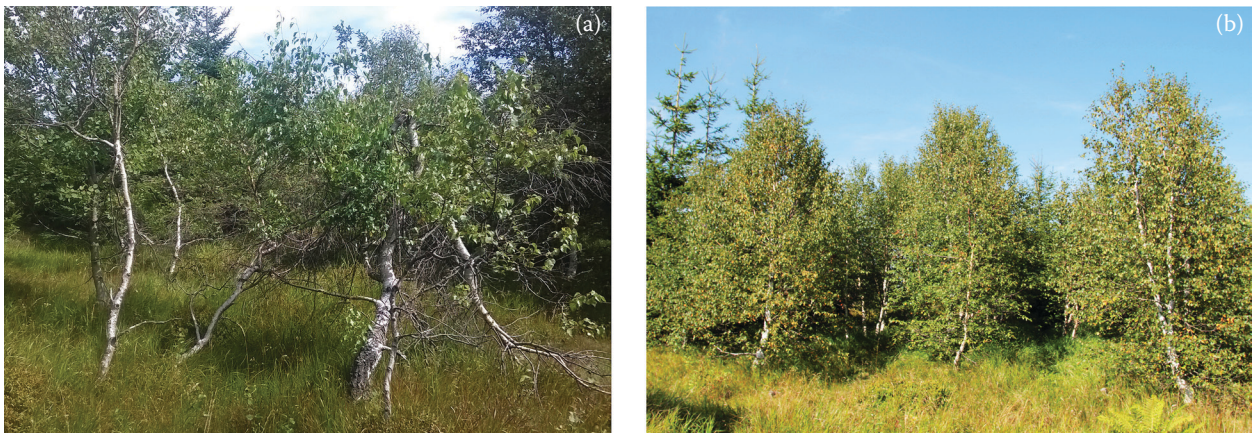

Fig. 1. Vitality of diploid (a) and tetraploid (b) birch taxa on the ridge of the Jizerské hory Mts. Stand age is 19 years. Photos were taken at the research plot managed by Forestry and Game Management Research Institute, Opočno Research Station (photo: R. Linda, M. Baláš)

lower altitudes (Podrázský, Ulbrichová 2004; Sułowicz et al. 2011).

In the Czech Republic downy birch is used in substitute stands on locations heavily disturbed in the past (i.e. by insects or air pollution), for example in the Jizerské hory Mts. These locations are situated at higher altitudes where downy birch, as one of the few tree species, can overcome heavy frosts, snow, and ice coating load (Fig. 1) (Kuneš et al. 2007).

Taxonomically, *B. pendula* and *B. pubescens* belong to the subgenus *Betula*, and section *Betula* (Ashburner, McAllister 2013). A simple cladogram of the *Betula* genus is shown in Fig. 2. Because of the close congeniality between these species, a reliable determination is possible only by holoploid genome size measurement in a laboratory with techniques such as flow cytometry (Suda, Pyšek 2010). Using flow cytometry is beneficial as *B. pendula* is diploid ($2n$ = 28) and *B. pubescens* is tetraploid ($2n$ = 56) (Gill, Davy 1983; Ashburner, McAllister 2013). Basic chromosome number of birches is x = 14 (Meier-Dinkel 1992).

In some cases, it is difficult to distinguish between diploid (*B. pendula*) and tetraploid (*B. pubescens*) birches in the field on the grounds of morphological traits (Hynynen et al. 2010). For forestry practice, nonetheless, it is useful to determine such species in the field by a simple and quick method accessible in terms of required instrumentation, as it was similarly proposed in previous studies (Gardiner, Jeffers 1962; Gardiner 1972; Atkinson, Codling 1986; Ešnerová et al. 2012).

The aim of this study is to suggest a reliable classification criterion for determining the diploid and

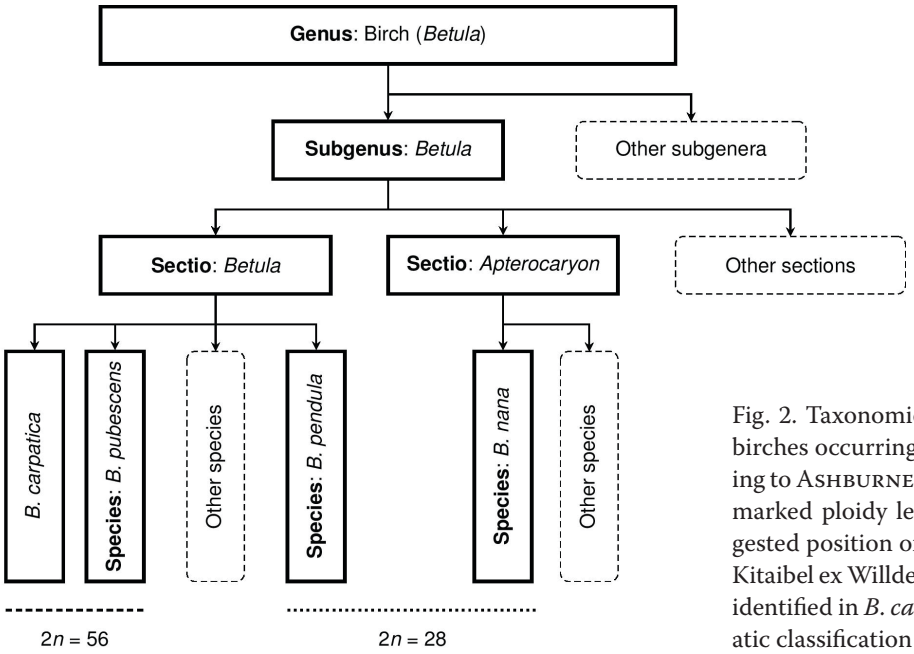

Fig. 2. Taxonomic treatment of selected taxa of birches occurring in the Czech Republic according to Ashburner and McAllister (2013) with marked ploidy level. Edited by R. Linda – suggested position of *Betula carpatica* Waldstein & Kitaibel ex Willdenow. The taxonomic rank is not identified in *B. carpatica* because of its problematic classification

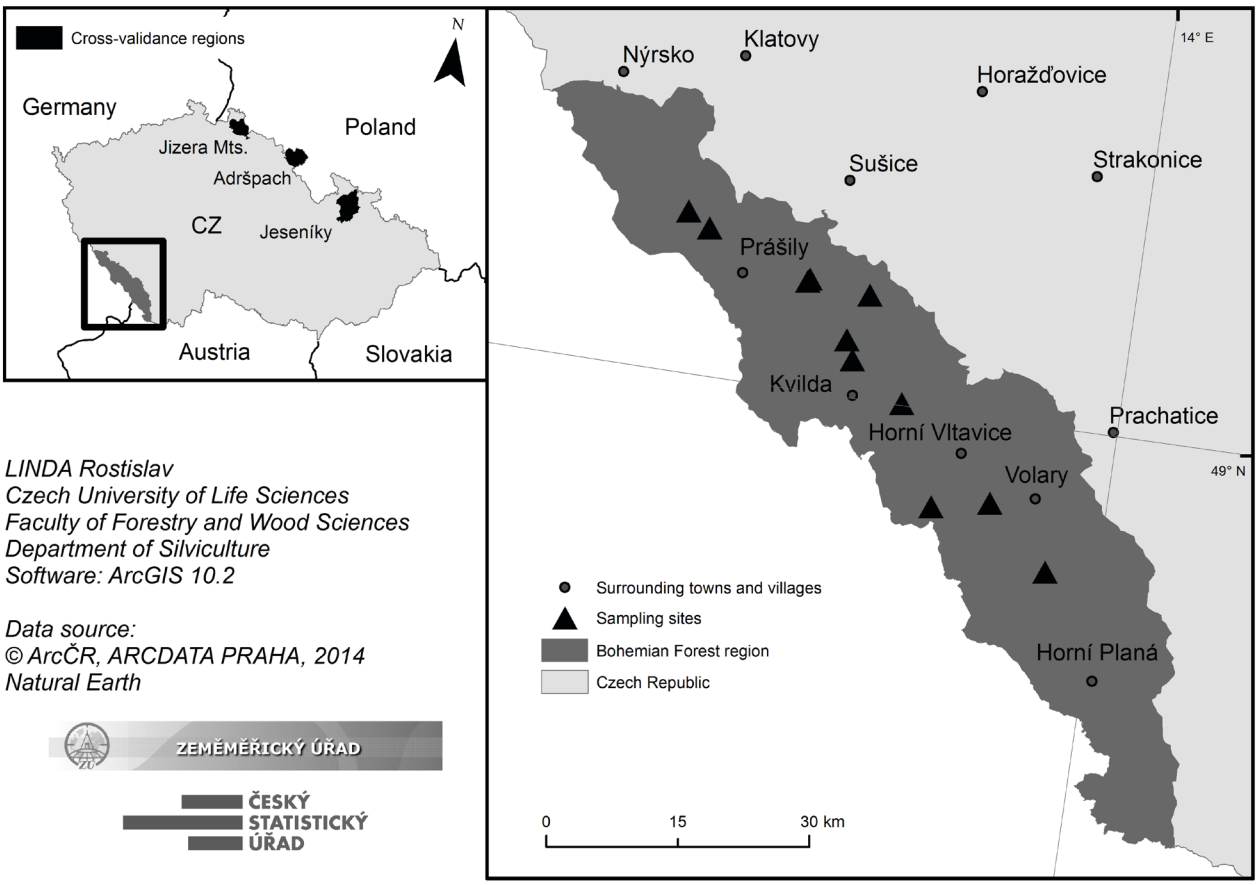

Fig. 3. Distribution of birch sampling sites

tetraploid taxa on chosen forest sites, distinguishing *B. pendula* from *B. pubescens*. The classification criterion should be built on macroscopic parameters, measurable with a common ruler or protractor to achieve simplicity. Additionally, the reliability of the proposed classification function was compared with similar criteria (functions) suggested by other authors.

MATERIAL AND METHODS

The sampling locations were situated in the Bohemian Forest region (Šumava Mts.). Distribution of sampling sites was based on tetraploid birch occurrences and with emphasis on maximum area coverage (Fig. 3).

A total of 97 individuals from 11 locations within the Bohemian Forest region were collected. At each location, 5–14 individuals were sampled. From each individual, 2 young branches with enough foliage were taken for morphometrics and a sufficient amount of leaves was used for the flow cytometry procedure (Suda, Pyšek 2010). Immediately after sampling, the material for propidium iodide flow cytometry was taken from the branches, placed into plastic bags in a refrigerated box and promptly transported for the analysis conducted in a laboratory of the Faculty of Science at Charles University in Prague. The branches were filed in the herbarium for subsequent morphological measurements.

Common daisy (*Bellis perennis* Linnaeus) was used as internal standard organism (2C = 3.38 pg) (Schönswetter et al. 2007) for flow cytometry. The samples together with the standard were chopped into Otto I buffer (Otto 1990). The suspension was stained with a solution containing 1 ml of Otto II buffer (Otto 1990), β-mercaptoethanol (2 $\mu l \cdot ml^{-1}$), propidium iodide (50 $\mu l \cdot ml^{-1}$) and RNase IIA (50 $\mu l \cdot ml^{-1}$). Prepared samples were run through a Partec CyFlow flow cytometer (Partec GmbH, Germany) with a green solid-state laser (Cobolt AB, Sweden; Cobolt Samba, 532 nm, 100 mW).

For statistical analysis, two leaves from each collected branch were measured (4 leaves per individual). On each leaf, 20 parameters were analysed – 16 quantitative (Fig. 4, Table 1) and 4 qualitative ones. These parameters were chosen according to previous studies (Gardiner, Jeffers 1962; Gardiner 1972; Gill, Davy 1983; Atkinson, Codling 1986). Except for the leaf base angles, the

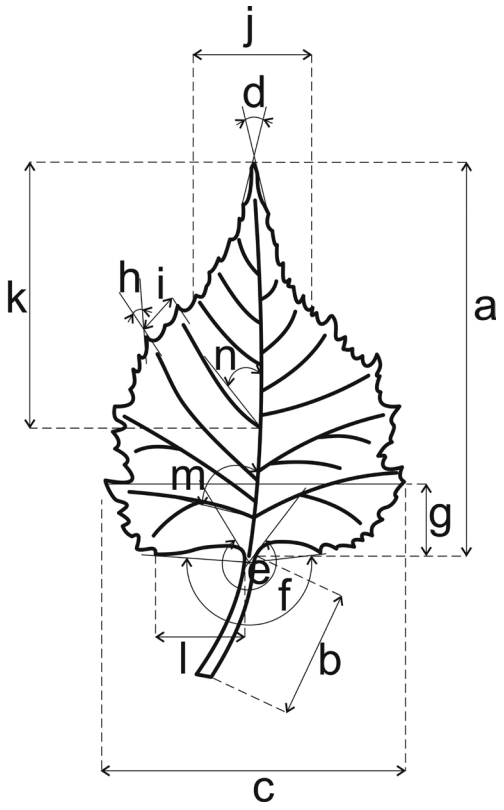

Fig. 4. Measured quantitative parameters. Two parameters are not displayed – i_1 and x (for details see Table 1)

parameters were measured on the left sides of the abaxial faces of the leaves. For leaf measurements, a ruler and a protractor were used; the measurement accuracy was 1 mm and 1°, respectively.

Four qualitative parameters were measured: vein arrangement (paired, unpaired), leaf base symmetry (symmetric, asymmetric), leaf base shape (distinctly heart-shaped, heart-shaped, round, straight, triangular), and leaf edge serration (simply serrate, doubly serrate, distinctly doubled, extremely doubly serrate).

Results of the flow cytometry and morphometric data were matched for further statistical analyses. The data for every single leaf were not averaged to preserve the variability within every single tree (every leaf was an observational unit). For general data structure overview, principal component analysis (PCA) was used. *t*-Test or Wilcoxon rank sum test (depending on data distribution) was used for the testing of differences between diploid and tetraploid taxa in each quantitative parameter.

The dependence of qualitative parameters on a ploidy level was tested either by χ^2 test of dependence in the contingency table or by Fisher's exact test, according to the number of distinguished levels. All statistical procedures were performed at a significance level of $\alpha = 0.05$.

Table 1. Description of measured quantitative parameters

Parameter	Description (unit)
a	blade length (mm)
b	petiole length (mm)
c	blade width (mm)
d	blade tip angle (°)
e	blade fitting angle (°)
f	basal angle (°)
g	distance of widest part of blade from blade base (mm)
h	leaf serration angle (°)
i	distance between 3rd and 4th vein (mm)
i_1	number of leaf teeth between 3rd and 4th vein
j	blade width in the upper 1/4 (mm)
k	distance from the 4th vein to the tip (mm)
l	distance from the leaf base to the 1st tooth (mm)
m	1st vein angle (°)
n	4th vein angle (°)
x	number of visible major leaf veins

For the prediction of actual ploidy level, the classification function was suggested. This function was computed via shrinkage discriminant analysis (SDA). Used parameters were selected by correlation-adjusted *t*-scores (CAT scores) (Zuber, Strimmer 2009). Classification functions with two to five parameters (the parameters were added to the function according to a descending CAT score) were tested for reliability. All computations were performed in the R environment (Version 1.3.7, 2016), concretely SDA was performed using the R package "SDA" (Ahdesmäki et al. 2015).

Overall performance of the suggested classification function was validated on individual data from three different regions in the Czech Republic – Jeseníky Mountains, Adršpach and Jizerské hory Mountains. These locations are enlisted in Florabase – Czech Portal of Botanical Data (Institute of Botany of the CAS 2010) as habitats of either diploid or tetraploid birch taxa and their environmental conditions are similar to those in the Šumava Mts. region (Chytrý et al. 2010).

RESULTS

According to the flow cytometry analyses, there were 13 diploid and 84 tetraploid individuals in the whole dataset. The PCA (Fig. 5) showed differences in leaf shapes between the diploid and tetraploid birches. The variability described by the first two principal components is 55.5%.

Testing of differences in each quantitative parameter showed significant results in 12 out of 16 traits (Table 2).

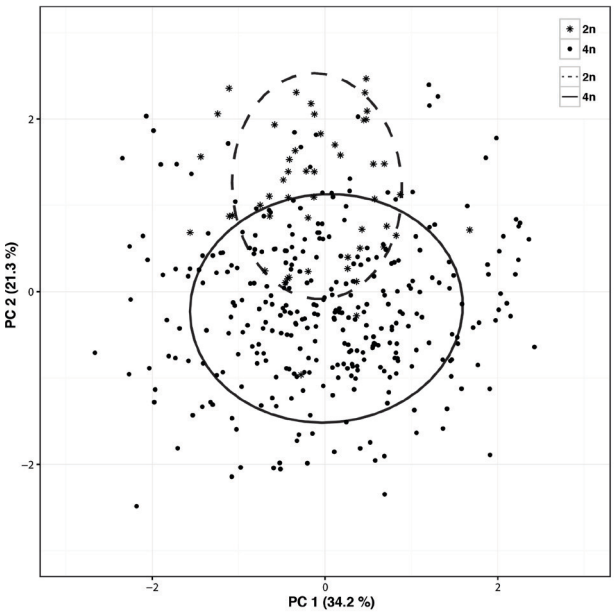

Fig. 5. Principal component analysis (PCA) of quantitative parameters. Each point depicts one measured leaf in space projected by the PCA method. Ellipses depict the most probable occurrence of individuals with the given ploidy level

PC 1, 2 – the first and the second principal component

As for qualitative data, 4 morphometric traits were analysed. Significant results were obtained for the parameter leaf base shape (χ^2, $P < 0.001$). Straight leaf base was the most common for diploid taxa – 36.5% of samples (95% confidence interval – 23, 50%). The most common leaf base shape of the tetraploid taxa was triangular – 65% of samples (95% confidence interval – 60, 70%).

For the individual ploidy prediction, a classification function (f) was designed (Eq. 1):

$$f = -9.627 - 0.449 \times j + 0.219 \times m + 1.729 \times i_1 \quad (1)$$

where:

j – blade width in the upper 1/4 (mm),

m – 1st vein angle (°),

i_1 – number of leaf teeth between the 3rd and 4th vein.

An individual is likely to be diploid, if $f > 0$ and conversely.

Three parameters were chosen into the classification function because its reliability showed a low increment with further added parameters (93% for 2 parameters, 96% for 3 parameters, 97% for 4 and 5 parameters).

Reliability of the classification function was tested on individuals from the three different locations of the Czech Republic, and compared with functions suggested by other authors (see the chapter Material and Methods). Overall reliability of the suggested criterion was 89% (Table 3).

Table 2. Testing of the differences between diploid and tetraploid taxa for each quantitative parameter. Values in the "Test" column describe the used statistical procedure, P – parametric (*t*-test), N – non-parametric (Wilcoxon rank sum test). The numbers in brackets in the "Mean" column depict minimum and maximum value of each parameter

Parameter	Test	Mean ($2n = 2x$)	Mean ($2n = 4x$)	*P*-value
a (mm)	P	42.3 (30, 55)	45.2 (22, 73)	0.004**
b (mm)	N	17.1 (10, 24)	15.8 (6, 29)	0.021*
c (mm)	P	34.5 (22, 46)	34.4 (15, 55)	0.908
d (°)	N	34.8 (18, 67)	40.8 (12, 81)	< 0.001***
e (°)	N	268.1 (196, 323)	272.4 (184, 334)	0.048*
f (°)	N	204.8 (138, 240)	233.2 (155, 295)	< 0.001***
g (mm)	N	12.7 (6, 21)	17.8 (7, 31)	< 0.001***
h (°)	N	66.6 (38, 126)	87.8 (11, 133)	< 0.001***
i (mm)	N	5.4 (4, 7)	5.2 (3, 9)	0.168
i_1 (–)	N	2.7 (1, 5)	1.4 (0, 4)	< 0.001***
j (mm)	N	11.8 (6, 17)	17.9 (7, 46)	< 0.001***
k (mm)	N	22.6 (11, 35)	21.2 (5, 41)	0.151
l (mm)	N	11.5 (6, 18)	11.1 (3, 24)	0.308
m (°)	N	55.2 (35, 73)	44.4 (27, 69)	< 0.001***
n (°)	N	31.1 (21, 42)	28.7 (12, 39)	< 0.001***
x (–)	N	6.8 (5, 9)	6.5 (4, 10)	0.038*

a – blade length, *b* – petiole length, *c* – blade width, *d* – blade tip angle, *e* – blade fitting angle, *f* – basal angle, *g* – distance of the widest part of blade from blade base, *h* – leaf serration angle (°), *i* – distance between 3rd and 4th vein, i_1 – number of leaf teeth between 3rd and 4th vein, *j* – blade width in the upper 1/4, *k* – distance from the 4th vein to the tip, *l* – distance from the leaf base to the 1st tooth, *m* – 1st vein angle, *n* – 4th vein angle, *x* – number of visible major leaf veins, statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 3. Reliability of compared classification functions on samples from selected regions of the Czech Republic

Region	Classification function reliability (%)			Samples in total
	this study	Atkinson and Codling (1986)	Ešnerová et al. (2012)	
Bohemian Forest	96	94	79	379
Jeseníky Mts.	87	92	80	131
Adršpach	82	76	74	158
Jizerské hory Mts.	84	85	81	268
Total	89	88	79	936

DISCUSSION

Taxonomic treatment within the genus *Betula*, as described earlier in the text, is still problematic. One approach to classifying birch taxa is by their ploidy level. Although this approach does not completely satisfy the requirements of the current taxonomic division (Ashburner, McAllister 2013), it can be very useful for forestry practice. As suggested e.g. by Balcar (2001), tetraploid birches can survive and perform significantly better than diploid *B. pendula* under specific extreme conditions, such as frost basins or mountain ridges in heavily air-polluted areas.

The distinction between diploid and tetraploid taxa is possible using laboratory techniques (e.g. flow cytometry), however, such applications are time-consuming and expensive. Classical morphometrics, together with the classification function, could be a fast and relatively reliable option.

Our results show that the leaf shapes of individuals with different ploidy differ in some parameters. Similar testing of each parameter was conducted previously by Ešnerová et al. (2012) with comparable results. PCA was done in both studies, while PCA in our work describes more differences in overall data structure.

The analysis of qualitative data did not show any patterns applicable to reliable ploidy prediction, though individuals with different ploidy showed differences in the most probable leaf base shape.

Our classification function showed very good reliability of primary data (96%) originating from the Bohemian Forest Mountains. According to analyses in this study the most suitable parameters for distinguishing between diploid and tetraploid birch taxa are blade width in the upper 1/4, 1st vein angle and number of leaf teeth between the 3rd and 4th vein. Leaf blade width was also used in studies by Atkinson and Codling (1986), and Ešnerová et al. (2012). Contrary to this study, a distance from the leaf base to the 1st tooth was used in both other mentioned studies, while this study uses the first vein angle instead. The last parameter used in this work was the number of leaf teeth between the 3rd and 4th vein, which was also used by Atkinson and Codling (1986), while Ešnerová et al. (2012) used the distance between the teeth of the 3rd and 4th vein.

The reliability level of the classification functions can naturally vary to some extent in various geographic regions. For example, all tested functions (Table 2) performed the worst in distinguishing the ploidy of individuals from Adršpach, where our function still performed the best from those compared. The function by Atkinson and Codling (1986) performed better on individuals from the Jeseníky Mts. and the Jizerské hory Mts. Another criterion, suggested by Ešnerová et al. (2012), showed ca. by 10% points lower overall reliability than those suggested in our study or by Atkinson and Codling (1986), but its reliability on samples from the Krkonoše Mts. was 100% according to the cited article.

Acknowledgment

Department of Botany, Charles University in Prague, for performing flow cytometry analyses for this study, RNDr. I. Bufková (Bohemian Forest Protected Landscape Area and Šumava National Park) for help with choosing suitable locations and Protected Landscape Areas Jeseníky, Adršpach and Jizerské hory Mountains.

Special thanks belong to M. Gutsch (SHARP Environmental Ltd.) for the language corrections.

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Received for publication July 24, 2017
Accepted after corrections November 3, 2017