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Taxonomic differentiation of *Salix retusa* agg. (Salicaceae) based on leaf characteristics

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Abstract

The complex of *Salix retusa* includes *S. retusa* s.s., *S serpyllifolia* and *S. kitaibeliana*, small, prostrate willows occurring in the subalpine and alpine vegetation belts of the mountains of Central Europe: the Pyrenees, Alps, Apennines, Dynaric Alps, Carpathians and Rila. The systematic positions of these taxa are not fully resolved and are still disputed. The aim of the present study was to biometrically verify differences in leaf characteristics between these taxa.

Material was collected from 47 populations, each represented by 25–52 individuals (33 on average). The study was based on 14 leaf morphological characters measured from scans using Win Folia software. The principal component analysis (PCA), Ward's agglomeration method, the *K*-means cluster analysis (*K*-MCA) and Kruskal-Wallis tests were applied to verify the relationships between taxa and their populations.

Differences between average leaf characteristics of *S. retusa* s.s., *S. serpyllifolia* and *S. kitaibeliana* were detected. The Pyrenean populations of *S. retusa* s.s. appeared more similar to *S. serpyllifolia*. Within the Alpine populations of *S. serpyllifolia*, several individuals resembling *S. retusa* s.s. were detected, and vice versa, within populations of *S. retusa* s.s., and there were also individuals similar to *S. serpyllifolia*. The controversial *S. kitaibeliana* was found to be typical of the Tatra Mountains.

The results support close taxonomic relations, but also the separate status of *S. retusa*, *S. serpyllifolia* and *S. kitaibeliana*. The differences between them are mainly of a quantitative nature.

Keywords: oreophytes, alpine plants, biometrics, morphology, systematics

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Introduction

The complex of *Salix retusa* L. includes *S. retusa* s.s., *S. serpyllifolia* Scop. and *S. kitaibeliana* Willd. (Rechinger, 1964; Dostál, 1989) (Supplementary Figs 1–4). *Salix serpyllifolia* has generally been treated as a separate species (e.g. Rechinger, 1957, 1964; Jalas & Suominen, 1976; Martini & Paiero, 1988; Skvortsov, 1999; Hörandl et al., 2002, and literature cited herein). However, *Salix kitaibeliana* has not only been considered a separate species, mostly in the local floras of the Carpathians (e.g. Szafer, 1921; Pawłowski, 1956; Koblížek, 2006; but also Rechinger, 1964), but it has also been treated as a subspecies, *S. retusa* subsp. *kitaibeliana* (Willd.) Jáv. (Baldie, 1952), and variety, *S. retusa* var. *kitaibeliana* (Willd.) Rchb., and finally, most frequently, as a synonym of *S. retusa* (e.g. Skvortsov, 1999; Mirek et al., 2002; Danilik, 2009).

The differences between these three taxa, independent of their systematic rank, are predominantly quantitative. *Salix serpyllifolia* has significantly smaller organs, while *S. kitaibeliana* has larger organs than *S. retusa* s.s. The leaves of *S. serpyllifolia* are less than half as long and wide as those of *S. retusa* s.s. (Rechinger, 1964) and the whole shrub is more compact, resembling 'miniaturised' *S. retusa* s.s. (Skvortsov, 1999). *Salix retusa* s.s. and *S. serpyllifolia* sometimes grow side by side in the Alps and usually the specimens can be easily distinguished in the field (Martini & Paiero, 1988; Skvortsov, 1999).

The leaves of *S. kitaibeliana* are more or less twice as long and broad as those of *S. retusa,* and their apex can sometimes be rounded, obtuse or even acute, not retuse (Rechinger, 1964; Dostál, 1989). However, in nature, many intermediate specimens between *S. retusa* s.s. and *S. kitaibeliana* can be observed, and the differences between these taxa are unclear (Pawłowski, 1956; Zieliński, 1977; Hardig et al., 2000). This has been the main reason for a lack of recognition between *S. retusa* and *S. kitaibeliana* (e.g. Zieliński, 1977; Skvortsov, 1999). The latter two taxa have been reported from different local environmental conditions in the Tatra Mountains: *S. retusa* s.s. is found on calcium-containing substrates, while *S. kitaibeliana* is found on siliceous rocks, mainly in moist places close to water sources and streams (Pawłowski, 1972; Dostál, 1989; Koblížek, 2006).

Salix retusa s.s. occurs in the mountains of Central Europe; from the Pyrenees in the West to the East Carpathians in the East and to the Central Apennines and Rila in the South. *Salix serpyllifolia* is considered as an Alpine subendemic species with only single localities in the Balkans, while the range of *S. kitaibeliana* is limited to the Carpathians (Pawłowski, 1972; Jalas & Suominen, 1976; Zając & Zając, 2009). All three taxa grow mainly in alpine zone, and sometimes in subalpine zone (Pawłowski, 1956, 1972; Baldie, 1967; Horvat et al., 1984; Ozenda, 1988; Martini & Paiero, 1988; Villar et al., 1997; Skvortsov, 1999; Hörandl et al., 2002; Ozenda & Borel, 2003; Zając & Zając, 2009). Their geographic distributions are discontinuous, divided into several parts depending on sufficient altitude of mountain massifs (e.g. Nagy et al., 2003; Ronikier, 2011). The populations of *S. retusa* s.s. in the Alps are isolated from the Carpathian, Balkan and Apennine ones (see Jalas & Suominen, 1976, figs 208 and 209). In addition, large distances separate populations within the Carpathians and Balkans. The distances between the Pyrenean, Alpine, Balkan and Carpathian ranges of *S. retusa* s.s. could be a reason for further morphological differentiation of this species, which has not been verified until now. The same may also have occurred for *S. retusa* and *S. serpyllifolia* in the Alps, as genetic break zones have been detected between West and East Alpine populations among species of the Alpine vegetation belts (Thiel-Egenter et al., 2011), and montane and subalpine tree species (Mosca et al., 2012).

Taking into account the above, we hypothesise that (1) morphological differences between leaf samples representing particular taxa of *S. retusa* group will be detected, (2) the Pyrenean, Alpine, Balkan and Carpathian populations of *S. retusa* s.s. will be morphologically different, (3) the populations of *S. retusa* s.s. and *S. serpyllifolia* sampled from the western versus eastern Alps could be morphologically different and (4) the populations of *S. retusa* s.l. (i.e. including *S. kitaibeliana*) sampled from calcareous sites will be morphologically different from those sampled from substrata of siliceous origin. The aim of the present study is to verify the above-mentioned hypotheses using biometrical analyses of leaves, organs most frequently found in herbarium collections, and thus validate the basis for distinguishing between *S. retusa* s.s., *S. serpyllifolia* and *S. kitaibeliana*.

Material and methods

Plant material collection and measurement procedure

Material was sampled from natural populations of *S. retusa* s.l. and *S. serpyllifolia*; *S. kitaibeliana* was not distinguished from *S. retusa* s.s. during sampling. Every individual was categorised in the field according to morphological characteristics accepted as taxonomically important for distinguishing between *S. retusa* s.s. and *S. serpyllifolia* (Rechinger, 1957, 1964; Skvortsov, 1999). Well-developed, uninjured leaves were collected from the central part of vegetative shoots. In order to avoid sampling the same genet more than once, sampled plants were separated by at least 10 m, as willows from the *S. retusa* group reproduce vegetatively by sprout rooting (the distance was sometimes smaller when neighbouring individuals were of opposite sex) (e.g. Rechinger, 1957; Hörandl et al., 2002; Kobližek, 2006). The 25–52 individuals (33 on average) from 15 populations of *S. serpyllifolia* and 32 populations of *S. retusa* s.l. (including possible *S. kitaibeliana*) were sampled (Table 1). In the Alps, *S. retusa* s.s. and *S. serpyllifolia* were sampled in eight cases in the same location. Every individual was represented by 5 leaves, which were dried in herbarium presses after sampling and stored in dry conditions until measurement.

Table 1. Sampled populations of *Salix retusa* agg.; N – number of sampled individuals

Code	Locality	N	Longitude [°]	Latitude [°]	Altitude [m]	Collector
A1	France, Alps, Maritime Alps, Auron	30	6.907	44.208	2268	PK, AH
A2*	France, Alps, Maritime Alps, Mt Bonette	35	6.833	44.335	2542	PK, AH
A3	France, Alps, Cottian Alps, Col de Vars	30	6.685	44.548	2395	PK, AH
A4*	France, Alps, Cottian Alps, Col d'Izoard	35	6.737	44.815	2293	PK, AH
A5	France, Alps, Cottian Alps, Col d'Izoard	30	6.735	44.820	2378	PK, AH
A6	France, Alps, Graian Alps, Col du Galibier	30	6.403	45.062	2599	PK, AH
A6*	France, Alps, Graian Alps, Col du Galibier	34	6.403	45.062	2599	PK, AH
A7a*	France, Alps, Graian Alps, Col de l'Iseran	35	7.042	45.401	2591	PK, AH
A7b*	France, Alps, Graian Alps, Val d'Isère	33	7.010	45.438	2447	PK, AH
A8	France, Alps, Graian Alps, Little St Bernard Pass	30	6.876	45.665	2193	PK, AH
A8*	France, Alps, Graian Alps, Little St Bernard Pass	32	6.876	45.665	2193	PK, AH
A9	Switzerland, Alps, Bernese Alps, Crans-Montana, las Violetes	30	7.498	46.342	2233	PK
A9*	Switzerland, Alps, Bernese Alps, Crans-Montana, las Violetes	31	7.498	46.342	2233	PK
A10*	Switzerland, Alps, Bernese Alps, Simplon Pass, Mt Hübschhorn	34	8.046	46.248	2167	PK
A11	Switzerland, Alps, Bernese Alps (Uri Alps), Furka Pass, Rhone Glacier	30	8.389	46.579	2384	PK, AH
A11*	Switzerland, Alps, Bernese Alps (Uri Alps), Furka Pass, Rhone Glacier	33	8.389	46.579	2384	PK, AH
A12*	Switzerland, Alps, Bernese Alps (Uri Alps), Oberalp Pass	25	8.673	46.660	2075	PK, AH
A13*	Switzerland, Alps, Pennine Alps, Great St. Bernard Pass	35	7.162	45.868	2401	PK, AH
A14	Switzerland, Alps, Glarus Alps, Pizol	30	9.434	46.980	2215	PK
A14*	Switzerland, Alps, Glarus Alps, Pizol	33	9.434	46.980	2215	PK
A15	Switzerland, Alps, Western Rhaetian Alps, Flüela Pass	30	9.941	46.752	2398	PK, AH
A16	Switzerland, Alps, Western Rhaetian Alps, Fuorn Pass (Ofen Pass)	30	10.293	46.639	2157	PK, AH
A17	Italy, Alps, Sarntaler Alpen, Königsangerspitze (Monte Pascolo)	30	11.581	46.708	2318	PK, AH
A17*	Italy, Alps, Sarntaler Alpen, Königsangerspitze (Monte Pascolo)	32	11.581	46.708	2318	PK, AH
A18	Italy, Alps, Dolomites, Rosengarten	30	11.617	46.416	2236	PK, AH
A18*	Italy, Alps, Dolomites, Rosengarten	31	11.617	46.416	2236	PK, AH
A19	Italy, Alps, Dolomites, Rolle Pass	30	11.795	46.308	2267	PK, AH
A20	Italy, Alps, Adamello-Presanella Alps, Mt Presanella	30	10.580	46.239	2573	PK
A20*	Italy, Alps, Adamello-Presanella Alps, Mt Presanella	35	10.580	46.239	2573	PK
A21	Italy, Alps, Adamello-Presanella Alps, Croce Domini Pass	30	10.414	45.915	2086	PK, AH
Ap1	Italy, Apennines, Abruzzi Apennines, Corno Grande	30	13.563	42.460	2473	PK
Ap2	Italy, Apennines, Sibillini Mts, Monte Vettore	30	13.281	42.822	2362	PK
B1	Bulgaria, Rila Mts, Rila Mts, Mt Mramorec	50	23.472	42.101	2567	PK
B2	Albania, Korab Massif, Korab Massif, Mt Korab	37	20.547	41.783	2596	PK
P2	Spain, Western Pyrenees, Bisaurín	39	-0,635	42,788	2559	PK, AH
P4	Spain, Western Pyrenees, Tendeñera Mts, Mt Sabocos	30	-0,263	42,687	2213	PK, AH
P5	Spain, Western Pyrenees, Pyrenees of Huesca, Bujaruelo valley	36	-0,077	42,702	2049	PK, AH
R1	Romania, Southern Carpathians, Bucegi Mts, Mt Jepii Mici	32	25.483	45.410	2079	PK
R2	Romania, Southern Carpathians, Făgăraș Mts, Balea Lake	30	24.616	45.600	2177	PK
R3	Romania, Southern Carpathians, Parâng Mts, Mt Cârja	30	23.521	45.365	2263	PK
R4	Romania, Eastern Carpathians, Rodna Mts, Mt Pietrosul Rodnei	30	24.638	47.595	2177	PK
T1	Slovakia, Western Carpathians, High Tatras, Vyšné Žabie pleso (tarn)	52	20.091	49.192	1723	PK, PG
T2	Slovakia, Western Carpathians, High Tatras, Zelené Kačacie pleso (tarn)	31	20.116	49.176	1595	PK, PG
T3	Poland, Western Carpathians, Western Tatras, Mt Beskid	35	19.980	49.235	1950	PK
T4	Poland, Western Carpathians, Western Tatras, Mt Jarząbczy Wierch	30	19.795	49.200	1737	PK, PG
T5	Slovakia, Western Carpathians, High Tatras, Sedlo pod Svišťovkou Pass	45	20.236	49.205	1920	PK, PG
T8	Poland, Western Carpathians, High Tatras, Black Lake below Mt Rysy	31	20.079	49.187	1575	PK

Asterix (*) – populations of *S. serpyllifolia*; PK – Piotr Kosiński, AH – Andreas Hilpold, PG – Piotr Górski.

Each leaf was characterised using 14 characteristics: 7 measured (Fig. 1B) and a further 7 resulting from calculations (Table 2). The leaf characteristics were chosen based on previous studies on the variation of *S. herbacea* L., *S. reticulata* L. and *Dryas octopetala* L. (Marcysiak, 2012a, 2012b, 2014) and from diagnoses of *S. retusa* group taxa (Pawłowski, 1956; Rechinger, 1957, 1974; Dostál, 1989; Koblížek, 2006). The measurement procedure follows those described by Jasińska et al. (2015) with implementation of WinFolia Pro 2003 (Regent Instruments Inc.) software.

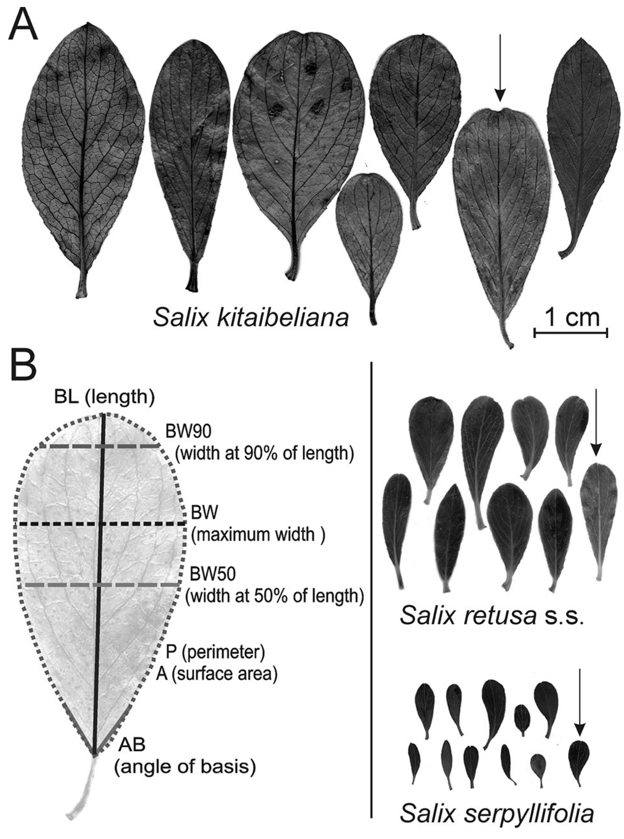

Fig. 1. A: examples of leaves of three willow taxa from *Salix retusa* group (arrows point to leaves close to average sizes); B: measured leaf characteristics

Statistical analyses

The frequency distribution of leaf characteristics values was verified using frequency histograms and the Shapiro-Wilk tests. To assess the possibility of parametric statistical test usage, the homoscedasticity of variances was verified by Brown-Forsythe test (Sokal & Rohlf, 2003). The data were standardised before further analyses to avoid the possible influence of variation resulting from the different types of characteristics (Sokal & Rohlf, 2003). For the statistical analyses, each individual was represented by an average of measurements of five leaves, and each population was represented by the average of all individuals.

The influence of particular characteristics on relationships among populations and the level of population differentiation was evaluated by implementation of principal component analysis (PCA) and Ward's agglomeration method based on Euclidean and Mahalanobis distances. These multivariate analyses were used to verify to which number of main clusters of populations, and subsequently of individuals, all data would be grouped and to which extent the populations/individuals would fit into taxonomic groups. The relationships among populations and individuals were illustrated on the scatter-plots of PCA, while clustering on dendrograms. A *K*-means cluster analysis (*K*-MCA) was performed as a supplementation of the agglomeration on the closest Euclidean and Mahalanobis distances. These analyses detected the number of *K*-clusters, which optimally illustrated the differentiation between populations. To determine the optimal *K* number, a scree plot was used with Δd value minimisation, when the succeeding nodes of agglomeration were added. Afterwards, a classification matrix was calculated to show the percentage of individuals within each population that were properly included into a particular *K* group (Sneath & Sokal, 1973). The *K*-grouping was presented cartographically to show geographic differentiation of the *S. retusa* complex.

The arithmetic mean (M), the median (MED), standard deviation (D) and variation coefficient (V) were calculated separately for every population and for each group detected with multivariate analysis, afterward comprehended as *S. retusa* s.s., *S. serpyllifolia* and *S. kitaibeliana*, to determine their ranges of diversity. The level of statistical significance of the differences between the median values of particular characteristics between taxa was verified using the Kruskal-Wallis test for data with skewed distribution and non-homoscedastic variances (Zar, 1999; Sokal & Rohlf, 2003). The STATISTICA 13 (Copyright © 2016 Dell Inc.) and JMP 12.1 (Copyright © 2015 SAS Institute Inc.) software were used for statistical analyses.

Results

Multivariate differentiation

The distribution of the analysed leaf characteristics generally had skewed distribution and were characterised with non homoscedastic variances. These circumstances did not allow use of the parametric tests. For this reason, we showed the medians of measurements beside the arithmetic means, we used PCA analysis for grouping the populations, and the Kruskal-Wallis test to verify the significance of differences between groups.

PCA divided all compared populations into three groups in the space between the three first canonical variables, which accounted for nearly 98% of the total variation (Fig. 2. 1–2). The right group was formed by populations sampled as *S. serpyllifolia* in the Alps, which differed from the central one, the *S. retusa* s.s. from the Pyrenees, Alps, Apennines, Balkans and the East and South Carpathians. The populations sampled as *S. retusa* s.l. in the Tatra Mountains all formed the left separate group, which was not as compact as the two previous ones (Fig.

Table 2. Average values of leaf characteristics of *Salix retusa*, *S. kitaibeliana* and *S. serpyllifolia*; M – arithmetic mean, MED – median, Min – minimum, Max – maximum, V – variation coefficient

Trait	Code	R - *Salix retusa*					K - *Salix kitaibeliana*					S - *Salix serpyllifolia*					Kruskal-Wallis test *p*		
		M	MED	Min	Max	V	M	MED	Min	Max	V	M	MED	Min	Max	V	R/K	R/S	K/S
Leaf blade surface area [cm²]	A	0.78	0.70	0.12	3.32	49.12	2.96	2.83	1.14	8.62	36.45	0.14	0.13	0.05	0.40	43.54	0.000	0.000	0.000
Leaf blade perimeter [cm]	P	3.67	3.58	1.26	7.23	27.44	7.65	7.65	4.64	11.51	16.96	1.51	1.45	0.78	2.80	23.90	0.000	0.000	0.000
Leaf blade length [cm]	BL	1.50	1.47	0.49	3.02	28.45	3.19	3.20	1.92	4.83	17.15	0.61	0.58	0.31	1.16	24.63	0.000	0.000	0.000
Leaf blade maximum width [cm]	BW	0.68	0.66	0.30	1.58	24.73	1.32	1.31	0.77	2.55	22.36	0.31	0.29	0.17	0.58	21.34	0.000	0.000	0.000
Leaf blade width at 50% of length [cm]	BW_50	0.64	0.62	0.29	1.52	24.63	1.23	1.21	0.71	2.42	23.28	0.29	0.28	0.17	0.57	20.74	0.000	0.000	0.000
Leaf blade width at 90% of length [cm]	BW_90	0.41	0.40	0.16	0.92	28.23	0.74	0.72	0.33	1.52	27.01	0.19	0.18	0.08	0.38	26.18	0.000	0.000	0.000
Angle of leaf blade basis [°]	AB	39.37	39.00	16.80	67.40	20.33	30.54	30.29	16.00	51.00	20.29	44.96	45.00	25.60	63.20	16.51	0.000	0.001	0.000
Leaf blade width at 90% / maximum width [100×(BW_50/BW)]	W_1	94.62	95.07	77.50	99.37	3.06	93.49	94.02	83.09	99.14	3.36	95.80	96.39	78.73	99.72	2.87	0.000	0.000	0.000
Leaf blade width at 50% / maximum width [100×(BW_90/BW)]	W_2	60.66	61.49	36.10	82.01	11.70	56.76	56.70	32.60	80.90	13.79	61.92	61.76	42.27	82.98	10.11	0.000	0.045	0.000
Leaf blade width at 90% / width at 50% of length [100×(BW_90/BW_50)]	W_3	64.48	64.62	36.85	99.37	13.72	61.17	60.88	33.60	96.50	15.93	64.94	64.02	43.28	111.03	12.55	0.000	1.000	0.000
Position of leaf maximal width [% of BL]	LBW	58.50	58.71	45.91	70.53	7.27	60.06	60.13	49.79	71.32	5.96	56.87	56.92	42.13	71.74	8.26	0.000	0.000	0.000
Ratio of leaf blade surface to circumference (A/P)	AP	0.20	0.19	0.09	0.45	23.41	0.37	0.37	0.22	0.76	22.07	0.09	0.09	0.06	0.16	19.43	0.000	0.000	0.000
Leaf blade length/width (BL/BW)	LW	2.25	2.20	1.23	3.81	19.49	2.50	2.50	1.49	4.47	17.73	2.01	1.96	1.24	3.26	17.57	0.000	0.000	0.000
Form coefficient ($4\times\varpi\times A/P^2$)	FC	0.70	0.70	0.38	1.07	15.95	0.63	0.61	0.36	4.19	40.65	0.77	0.78	0.48	1.03	13.34	0.000	0.000	0.000

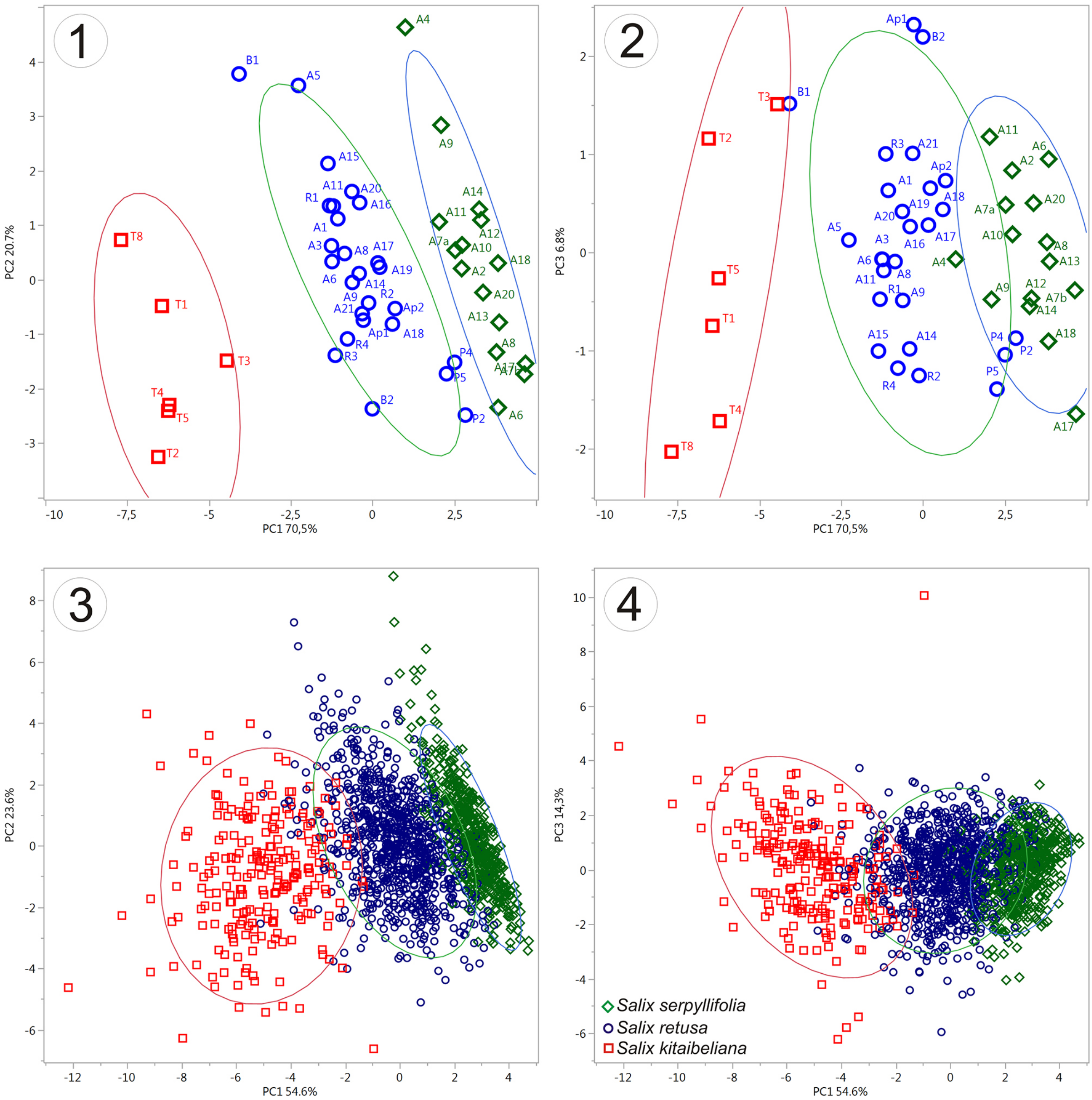

Fig. 2. Morphological subdivision of *Salix retusa* complex into three groups: *S. retusa* s.s., *S. serpyllifolia* and *S. kitaibeliana*: results of PCA for populations (1–2) and for individuals (3–4); lines indicate 90% confidence intervals for each group of individuals (codes of populations as in Table 1)

2. 1–2). We provisionally named this group *S. kitaibeliana*, because the values of dimensional characters (A, P, BL, BW) were the highest when compared to the other populations. The first canonical variable was determined mostly by the leaf dimensional characters (A, P, BL, BW, BW50 and BW90) and position of the leaf maximal width (LBW), the second by ratios of leaf widths (W2 and W3) and leaf area to the circumference (AP), while the third by the ratio of leaf length to width (LW).

The result of PCA conducted on the level of individuals also showed three clouds of individuals representing *S. serpyllifolia*, *S. retusa* s.s. and *S. kitaibeliana*, but very close to each other and partly intermingled. Surprisingly, the individuals of *S. serpyllifolia* substantially entered the 95% confidence interval of *S. retusa* s.s., while individuals of *S. kitaibeliana* intermingled with those of *S. retusa* s.s. to a lesser degree (Fig. 2. 3–4). The differences between taxa are determined mostly by the first canonical variable, while the second and third canonical variables showed differences between individuals within a taxa.

The clustering on the shortest Euclidean distances between populations also showed differentiation of populations to the three main groups. The populations of *S. retusa* s.s. sampled in the Alps, Apennines, Balkans and East and South Carpathians formed one cluster. Within this group, populations from different geographic regions were intermingled and did not reveal any geographic pattern of differentiation

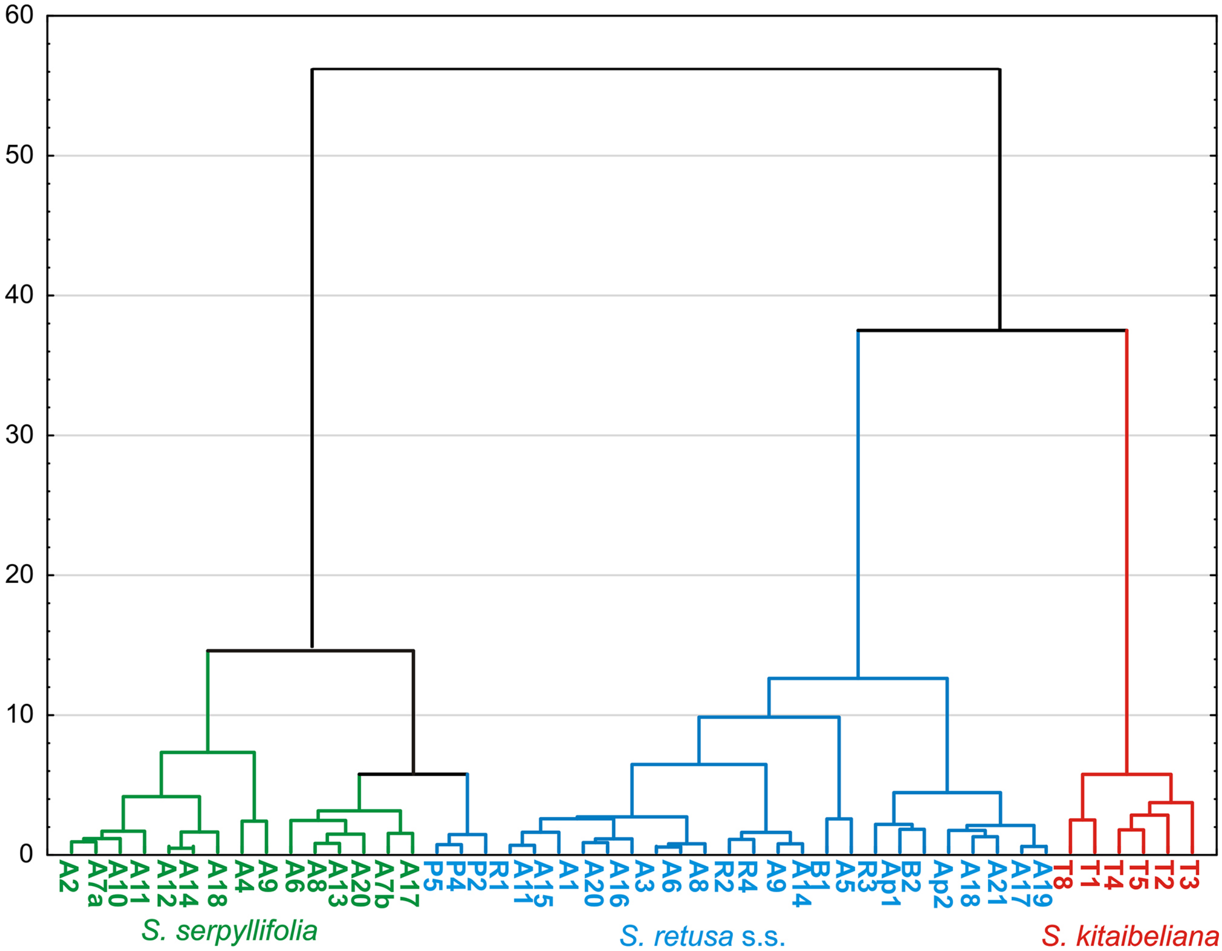

Fig. 3. Dendrogram constructed on Euclidean shortest distances between populations using Ward's method (populations codes as in Table 1)

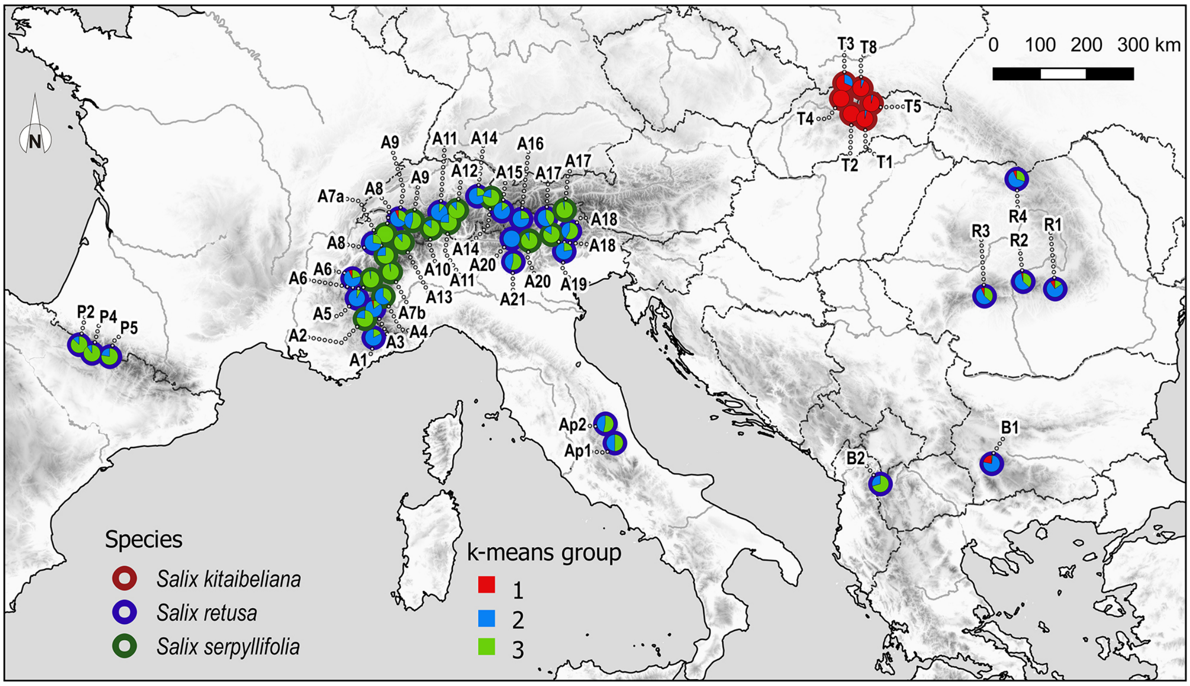

Fig. 4. Geographic distribution of three groups of populations of the *Salix retusa* complex detected with *K*-means clustering. Symbols indicate the percentage proportions of individuals included in one of the three *K*-groups within every sampled population

(Fig. 3). The second cluster, provisionally named *S. kitaibeliana*, formed populations from the Tatra Mountains. The third cluster was formed by populations of *S. serpyllifolia*, but also included three Pyrenean populations of *S. retusa* s.s. as a separate sub-cluster. Population differentiation on the basis of the Mahalanobis distances was similar to that found on the basis of Euclidean distances (data not shown).

The three groups of populations detected by PCA and confirmed in the clustering were also detected as optimal when the *K*-grouping method was applied. However, the classification matrix showed that most of the analysed populations included individuals from two or even three groups (Fig. 4). Surprisingly, the individuals classified as *S. serpyllifolia* prevailed in populations from the Pyrenees and individuals resembling *S. kitaibeliana* were detected in the Balkan and Alpine populations.

The differences between *S. retusa* s.l. (including *S. kitaibeliana*) populations sampled from calcareous versus siliceous sites have not been detected. The statistically significant differences were found only between populations sampled from the siliceous rocks in the Tatra Mountains and the other populations in the entire data set of *S. retusa* s.s.

Differentiation of characteristics

The most variable characteristics were the area of leaf blade (A) and the leaf form coefficient (FC), both with variation coefficients higher than 40% in *S. retusa* s.s. and *S. serpyllifolia* (A), and in *S. kitaibeliana* (FC) (Table 2). The average of variation coefficients for *S. retusa* s.s., *S. kitaibeliana* and *S. serpyllifolia* were 21.2, 20.2 and 18.6%, respectively (Table 2). The dimensional characteristics were statistically significantly correlated ($p \leq 0.01$), with $r = 0.90$ or higher.

Every characteristic, except for the ratio of leaf blade width at 90% to width at 50% of the length (W_3), and leaf blade width at the mid-length to maximal width (W_2) differed significantly ($p \leq 0.01$) between possible combinations of the three taxa (Table 2). W_2 differed between *S. retusa* s.s. and *S. kitaibeliana* ($p \leq 0.01$), between *S. kitaibeliana* and *S. serpyllifolia* ($p \leq 0.01$), and between *S. retusa* s.s. and *S. serpyllifolia* ($p \leq 0.05$). W_3 did not differ between *S. retusa* s.s. and *S. serpyllifolia*. Despite significant differences between average values of dimensional features, the variation ranges of shape characteristics overlapped significantly (Table 2).

Discussion

All 14 characteristics describing leaf morphology separated *S. retusa* s.s. from *S. kitaibeliana*, and *S. kitaibeliana* from *S. serpyllifolia*, while 13 characteristics distinguished *S. retusa* s.s. from *S. serpyllifolia*. These results are consistent with the accepted taxonomic classification of *S. retusa* s.s. and *S. serpyllifolia* (e.g. Neumann, 1981; Chmelař et al., 1979; Dostál, 1988; Martini & Paiero, 1988; Skvortsov, 1999; Hörandl et al., 2002). The very high level of distinction of *S. kitaibeliana* from *S. retusa* s.s. found in the present study (Table 2, Figs. 1 and 2) confirms its separate taxonomic status proposed at least for the Tatra Mountains (Pawłowski, 1956; Rechinger, 1957, 1964; Dostál, 1989; Koblížek, 2006). The combination of dimensional and shape characteristics of leaves allowed differentiation with a high probability between *S. retusa* s.s., *S. serpyllifolia* and *S. kitaibeliana*, despite partly overlapping variation ranges of particular characteristics among studied taxa (Table 2).

The leaves of *S. retusa* s.s. were found as 0.5–3.0 cm long (1.5 cm on average) and 0.3–1.5 cm wide (0.7 cm on average), while those of *S. serpyllifolia* were 0.3–1.2 cm long (0.6 cm on average) and 0.2–0.6 cm wide (0.3 cm on average) (Fig. 1A). These data correspond well with those reported in the literature (compare Tables 2 and 3). It should be noted, however, that average values of BL and MW reported from the Tatra Mountains (e.g. Zapałowicz, 1908; Szafer, 1921; Pawłowski, 1956; Koblížek, 2006) were much higher than from other parts of the geographic range of *S. retusa* s.s. (Table 3). The close systematic relationships between taxa of the *S. retusa* complex have been stressed by Rechinger (1957, 1964), who distinguished *S. retusa*, *S. serpyllifolia* and *S. kitaibeliana*. The lack of differences between *S. retusa* s.s. and *S. kitaibeliana* has been underlined by Skvortsov (1999). This could result from the limited number of herbarium materials from the Tatra Mountains that were accessible for him. The data presented here result from analyses of a large number of individuals and thus, for the first time, give exact and reliable information on not only commonly reported leaf dimensional characteristics, the most frequently used of which is length and width (BL and BW, respectively in Table 2), but also five other morphological traits, not reported until now. From the 224 individuals of *S. retusa* s.l. sampled in the Tatra Mountains, 210 had leaf characteristics that classified them as *S. kitaibeliana* (Fig. 4).

Every population sampled from the Tatra Mountains had leaf length and widths twice as high as the average found for all populations of *S. retusa* s.s. (Table 2). This finding could be interpreted as resulting from the separate geographic position within the geographic range of *S. retusa* s.l., but also as having taxonomic status. Unfortunately, the populations from the Tatra Mountains have only been sampled from siliceous substrata (Table 1), which was designated as typical sites of *S. kitaibeliana* (Pawłowski, 1956, 1972; Zieliński, 1977; Koblížek, 2006). Nevertheless, the

Table 3. Morphological comparison of *Salix retusa* s.s., *S. serpyllifolia* and *S. kitaibeliana* based on data reported in literature

Plant character	*Salix retusa* s.s.	Salix serpyllifolia	Salix kitaibeliana
Height [cm]	5–20 (–40) (Martini & Paiero, 1988)	No more than 1–3 (Martini & Paiero, 1988)	up to 20 cm (Kobližek, 2006)
	1–10 (Hörandl et al., 2002)	1–2 (Hörandl et al., 2002)	Up to 50 (Dostál, 1989)
	10–30 (Blanco P. 1993)		
	Up to 30 (Baldie, 1952; Dostál, 1989)		
	Up to 39 (Jovanović & Tucović, 1972)		
branches [cm]	Long (Rechinger, 1957, 1964)	very short (Rechinger, 1957)	Up to 60 (Pawłowski, 1956)
	Up to 30 (Pawłowski, 1956)		
Leaf length [cm]	1.0–1.5 (–3.3) (Rechinger, 1957)	0.3–1.1 (Rechinger, 1957)	2.5–4.0 (–5.0) (Dostál, 1989)
	1.2–2.0 (–2.5) (Dostál, 1989)	0.4–0.8 (–1.0) (Martini & Paiero, 1988)	2.5–5.0 (–7.0) (Kobližek, 2006)
	1.5–5.0 (Kobližek, 2006)	0.4–1.0 (Rechinger, 1964)	2.0–3.5 (Rechinger, 1964)
	(0.8–) 1.0–2.0 (–4.0) (Martini & Paiero, 1988)	0.4–0.8 (Hayek, 1927)	Largest (2.0–) 2.5–4.0 (–5.0) (Pawłowski, 1956)
	0.8–2.0 (Rechinger, 1964)	0.2–0.8 (Hess et al., 1967)	3.5–4.5 (Zapałowicz, 1908)
	1.5–3.5 (Hayek, 1927)	0.4–1.0 (Fenaroli, 1971)	1.5–3 (–3.5) (Baldie, 1952)
	1.0–2.0 (Szafer, 1921)		
	1.5–3.0 (Zapałowicz, 1908)		
	1.0–2.0 (Hess et al., 1967)		
	0.8–2.0 (Fenaroli, 1971)		
	0.5–2.5 (Jovanović & Tucović, 1972)		
	(0.5–) 0.8–3.0 (–3.5) (Baldie, 1952)		
	2.0 (Blanco, 1993)		
	Largest 1.2–2.0 (–2.5) (Pawłowski, 1956)		
Leaf width [cm]	0.5–0.7 (1.6) (Rechinger, 1957)	0.2–0.4 (Rechinger, 1957, 1964)	1.5–2.5 (–3.0) (Kobližek, 2006)
	0.5–1.5 (Kobližek, 2006)	0.2–0.4 (Martini & Paiero, 1988)**	0.7–1.1 (Rechinger, 1964)
	0.5–1.5 (–2.0) (Martini & Paiero, 1988)**	0.3–0.3 (Hayek, 1927)	1.2–1.8 (Zapałowicz, 1908)
	0.5–08 (Rechinger, 1964)	0.1–0.4 (Hess et al., 1967)**	
		0.4–0.4 (Fenaroli, 1971)	
	1.0 (Blanco, 1993)		
Inflorescence length × diameter [mm]	15 × 5 (Rechinger, 1957)***	5 × 5 (Rechinger, 1957)	(15–) 20–40 (–55) (Pawłowski, 1956)
	20–25 × 5–10 (Kobližek, 2006)*	5 (Hörandl et al., 2002)*	
	10–20 (–25) (Pawłowski, 1956)		
	10–20 (Hörandl et al., 2002)*	5 (Martini & Paiero, 1988)	
	15–30 × 10 (Zapałowicz, 1908)		
	Up to 2.0 (Baldie, 1952)		
	15–20 (Martini & Paiero, 1988)		
Flower number in catkin	More than 10 (Martini & Paiero, 1988)***	5–7 (Rechinger, 1957)	20–40 (Kobližek, 2006)
	8–20 (Pawłowski, 1956)	2–7 (Martini & Paiero, 1988)	20–50 (or more) (Pawłowski, 1956)

* – without distinguishing between male and female catkin.
** – after calculation from leaf length/width ratio.
*** – only male catkins.

Romanian populations (Eastern and Southern Carpathians) growing on acid rocks displayed leaf characteristics mostly similar to *S. retusa* s.s. It is noteworthy, that five of the six analysed populations from the Tatra Mountains contained only the typical *S. kitaibeliana* individuals, or included single individuals resembling *S. retusa* s.s. (Fig. 4). Populations from the Eastern and Southern Carpathian Mountains (Romania) and Rila (Bulgaria) included some amount of *S. kitaibeliana* individuals. This is similar to that found in subalpine *Pinus mugo* Turra populations, where some individuals typical of the Tatra Mountains were detected in populations from the East and South Carpathians and Bulgarian Mountains (Boratyńska et al., 2015; Dzialuk et al., 2016).

The differences in leaf traits between *S. retusa* s.s., *S. serpyllifolia* and *S. kitaibeliana* have mostly been of quantitative nature. This has been stressed by most authors (e.g. Pawłowski, 1956, Rechinger, 1957, 1964; Kobližek, 2006). For this reason, all three taxa were grouped as *S. retusa* (Rechinger, 1964). Our results support this opinion.

The marginal populations sampled in the Apennines and in the Albanian Korab Massif contained an amount of individuals classified as *S. serpyllifolia* (Fig. 4), i.e. with smaller leaves than are found for typical *S. retusa*. This could be a result of long isolation of marginal populations and from more xeric conditions. This may be confirmed by the fact that some individuals from these populations growing in moist conditions developed longer shoots and larger leaves (Supplementary Fig. 4). The influence of habitat conditions on the growth of these willows should be verified in the additional study.

A further new finding of the present study is the different position of *S. retusa* from the Western Pyrenees. The three populations from this mountain range appeared to be different from the Alpine and Carpathian populations and were even grouped together with the Alpine population of *S. serpyllifolia* (Fig. 3). The result of the PCA conducted on the level of population (Fig. 2) showed that the Pyrenean populations were placed between *S. retusa* s.s. and *S. serpyllifolia*. This was also confirmed by the Kruskal-Wallis test (data not included) performed for three groups of populations (*S. serpyllifolia, S. retusa* s.s. excluding Pyrenees, and Pyrenean populations of *S. retusa*), where populations from the Pyrenees differed significantly both from *S. serpyllifolia* (13 characteristics) and *S. retusa* s.s. (all characteristics). The Pyrenean populations were characterised by intermediate size of leaves (Supplementary Figs 5–6) and more rounded leaf blades (Supplementary Figs 7–8). These results may indicate a separate taxonomical status of Pyrenean populations of *S. retusa,* but this should be confirmed in a deeper study.

Conclusion

The results support close taxonomic relationships, but also the separate status of *S. retusa*, *S. serpyllifolia* and *S. kitaibeliana*.

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References

Baldie A (1952) Salicaceae: Flora Republicii Populare Romane, vol. 1 (ed. by T Săvulescu) Academia Republicii Populare Române, pp. 265–322.

Blanco P (1993) *Salix* L.: Flora Iberica, vol. 3 (ed. by S Castroviejo, C Aedo, S Cirujano, M Laínc, P Montserrat, R Morales, F Muñoz Garmendia, C Navarro, J Paiva & C Soriano) Madrid: Real Jardín Botánico, C.S.I.C., pp. 477–517.

Boratyńska K, Jasińska AK & Boratyński A (2015) Taxonomic and geographic differentiation of *Pinus mugo* complex on the needle characteristics. Systematics and Biodiversity 13: 581–595.

Chmelař J, Meusel W, Lattke H & Hammerling H-J (1979) Die weiden Europas: die gattung *Salix*. 2 ed. A. Ziemsen Verlag, Wittenberg Lutherstadt.

Danilik IM (2009) *Salix retusa* L.: Chervona kniga Ukrainy (ed. by YP Didukh) Kyiv, Globalkonsalting, p. 587.

Dostál J (1989) Nová květena ČSSR, 1. Academia, Praha.

Dzialuk A, Boratyńska K, Romo A & Boratyński A (2016) Taxonomic and geographic variation of the *Pinus mugo* complex on chloroplast microsatellite markers. Systematics and Biodiversity. doi:10.1080/14772000.2016.1257518.

Fenaroli L (1971) Flora delle Alpi. 2nd ed. Aldo Martello, Milano.

Hardig TM, Brunsfeld SJ, Fritz RS, Morgan M & Orians CM (2000) Morphological and molecular evidence for hybridization and introgression in a willow (*Salix*) hybrid zone. Molecular Ecology 9: 9–24.

Hayek A (1927) Prodromus florae peninsulae Balkanicae, 1. Repertorium speciorum novorum regni vegetabilis, Beihefte 30.1.

Hess HE, Landolt E & Hirzel R (1967) Flora der Schweiz, 1. Birkhäser, Basel und Stuttgart.

Horvat I, Glavač V & Ellenberg E (1984) Vegetation Südeuropas. Geobotanica Selecta 4. G. Fischer, Stuttgart.

Hörandl E, Florineth F & Hadacek F (2002) Weiden in Österreich und angrenzenden gebieten. Universität für Bodenkultur, Wien.

Jalas J & Suominen J (1976) Atlas florae Europaeae: distribution of vascular plants in Europe Vol. 3 Salicaceae to Balanophoraceae. The Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo, Helsinki.

Jasińska AK, Rucińska B, Kozlowski G, Bétrisey S, Safarov H, Boratyńska K & Boratyński A (2015) Morphological differentiation of leaves in the relict tree *Zelkova carpinifolia* (Ulmaceae). Dendrobiology 74: 109–122.

Jovanović B & Tucović A (1972) Salicaceae: Flore de la Republique Socialiste de Serbie, 3 (ed. by M Josifović) Academie Serbe des Sciences et des Arts, Beograd, pp. 405–457.

Koblížek J (2006) *Salix* L.: Flóra Slovenska, v. 3 (ed. by K Goliašová & E Michalková) Veda, Bratislava, pp. 209–290.

Marcysiak K (2012a) Variation of leaf shape of *Salix herbacea* in Europe. Plant Systematics and Evolution 298: 1597–1607.

Marcysiak K (2012b) Diversity of *Salix reticulata* L. (Salicaceae) leaf traits in Europe and its relation to geographical position. Plant Biosystems 146, Suppl. 1: 101–111.

Marcysiak K (2014) Geographical differentiation of *Dryas octopetala* in Europe based on morphological features. Dendrobiology 72: 113–123.

Martini F & Paiero P (1988) I salici d'Italia: Guida al riconoscimento e all'utilizzazione pratica. Trieste, Edizioni LINT.

Mirek Z, Piękoś-Mirkowa H, Zając A & Zając M (2002) Flowering plants and pteridophytes of Poland. A checklist W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków.

Mosca E, Eckert AJ, Di Pierro EA, Rocchini D, La Porta N, Belletti P & Neale DB (2012) The geographical and environmental determinants of genetic diversity for four alpine conifers of the European Alps. Molecular Ecology 21: 5530–5545. doi: 10.1111/mec.12043.

Nagy L, Grabherr G, Körner Ch & Thompson DBA (2003) Alpine biodiversity in Europe. Berlin – Heidelberg, Springer.

Neumann A (1981) Die mitteleuropäischen *Salix* Arten. Österreich. Agrarverlag, Wien.

Ozenda P (1988) Die Vegetation der Alpen im europäischen Gebirgsraum. Elsevier, München.

Ozenda P & Borel J-L (2003) The Alpine Vegetation of the Alps: Alpine biodiversity in Europe (ed. by L Nagy, G Grabherr, Ch Körner & DBA Thompson) Ecological Studies, Springer, Berlin, Heidelberg, pp. 53–64.

Pawłowski B (1956) Flora Tatr. Rośliny naczyniowe. Vol. 1. Państwowe Wydawnictwo Naukowe, Warszawa.

Pawłowski B (1972) Szata roślina gór Polski: Szata roślinna Polski, 2 (ed. by W Szafer & K Zarzycki) Polskie Wydawnictwo Naukowe, Warszawa, pp. 189–250.

Rechinger KH (1957) *Salix* L.: Illustrierte flora von Mitteleuropa Bd. 3.1. 2nd ed. (G Hegi) C. Hanser, München, pp. 44–135.

Rechinger KH (1964) *Salix* L.: Flora Europaea 1 (ed. by TG Tutin, VH Heywood, NA Burges, DH Valentine, SM Walters & DA Webb) University Press, Cambridge, pp. 45–54.

Ronikier M (2011) Biogeography of high-mountain plants in the Carpathians: An emerging phylogeographical perspective. Taxon 60: 373–389.

Skvortsov AK (1999) Willows of Russia and adjacent countries. Joensuu University Press, Joensuu, Finland.

Sneath PH & Sokal RR (1973) Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman, San Francisco, CA.

Sokal RR & Rohlf FJ (2003) Biometry. The principles and practice of statistics in biological research. 3rd ed. W. H. Freeman and Co., New York.

Szafer W (1921) Salicales: Flora polska, 2 (ed. by W Szafer) Akademia Umiejętności, Kraków, pp. 24–47.

Thiel-Egenter C, Alvarez N, Holderegger R, Tribsch A, Englisch T, Wohlgemuth T, Coli L, Gaudeul M, Gielly L, Jogan N, Linder HP, Negrini R, Niklfeld H, Pellecchia M, Rioux D, Schönswetter P, Taberlet P, van Loo M, Winkler M, IntraBioDiv Consortium & Gugerli F (2011) Break zones in the distributions of alleles and species in alpine plants. Journal of Biogeography 38: 772–782. doi:10.1111/j.1365-2699.2010.02441.x.

Villar PL, Sesé JA, Franco JAS & Ferrández JV (1997) Atlas de la flora del Pirineo Aragonés. Vol. 1. Consejo de Protección de la Naturaleza de Aragón, Huesca.

Zając M & Zając A (2009) The geographical elements of the native flora of Poland. Laboratory of Computer Chorology, Institute of Botany, Jagiellonian University, Kraków.

Zapałowicz H (1908) Conspectus florae Galiciae criticus. Akademia Umiejętności, Kraków.

Zar JH (1999) Biostatistical analysis. Prentice Hall, New Jersey.

Zieliński J (1977) *Salix retusa* L.: Atlas of distribution of trees and shrubs in Poland, vol. 22 (ed. by K Browicz) pp. 7–9, 41–42.