

Inference of taxonomic relationships between *Rhododendron ferrugineum* and *R. myrtifolium* (Ericaceae) from leaf and fruit morphologies

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Rhododendron myrtifolium and *R. ferrugineum* are small, shrubby plant species that are morphologically similar to each other. They both grow on siliceous substrates in the subalpine belt of European mountains. In the present study, leaves and capsules from 11 populations of *R. myrtifolium* from the Carpathians were compared with those from 13 populations of *R. ferrugineum* from the Alps, Pyrenees and Sudetes. Fourteen leaf characteristics and 12 capsule characteristics were analysed using ANOVA, discrimination analysis, principal component analysis and cluster analysis with agglomeration and the K-means method. All of the tested characteristics differed significantly between species, with the highest level of discrimination from the measured leaf traits. Less discriminatory were the proportions of the measured leaf features. According to the analyses, the populations of the two species were clearly separated, but individuals were partially intermixed. The similarities of the species may indicate a late divergence event, probably no earlier than the Pliocene. During the glacial cycles of the Pleistocene, populations of the two species may have mixed again. This may explain the presence of single individuals with *R. myrtifolium* morphology in some alpine *R. ferrugineum* populations and vice versa. The two species can be treated as another example of Carpathian–Alpine vicariance.

ADDITIONAL KEYWORDS: Alps – Carpathians – discrimination analysis – plant migration – plant variation – Pyrenees – taxonomy.

INTRODUCTION

Rhododendron L., with > 1000 species (Chamberlain *et al.*, 1996), is one of the most species-rich genera of woody plants (Khan *et al.*, 2021). The genus probably arose in north-eastern Asia in the early Palaeocene (Shrestha *et al.*, 2018a). The greatest current diversity of *Rhododendron* spp. is in south-western Asia, and they also occur in other parts of Asia, North America, Europe and Australia (Heywood, 1978; Fang *et al.*, 2005; Judd & Kron, 2009; Shrestha *et al.*, 2018a).

The large number of species, morphological variability and extensive range explain the many studies on the

taxonomy of the genus. The infrageneric system for the genus, with subgenera and sections, was formed on the basis of morphological characters, such as deciduous versus evergreen leaves, the presence of lepidote scales on the leaves and floral characters (e.g. Davidian, 1982; Chamberlain *et al.*, 1996; Cullen, 2005; Xia *et al.*, 2022). Molecular phylogenetic studies are, to some extent, in accordance with the classifications based on morphology, but there are also some incongruences (Goetsch *et al.*, 2005; Khan *et al.*, 2021).

The most abundant subgenus, *Rhododendron* subgenus *Rhododendron*, is distinguished in most systematic approaches, although its internal taxonomic division is the subject of discussion (Khan *et al.*, 2021; Xia *et al.*, 2022). The evergreen *Rhododendron*

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ferrugineum L., *Rhododendron myrtifolium* (Schott & Kotschy) and *Rhododendron hirsutum* L. belong to *Rhododendron* section *Rhododendron* subsection *Rhododendron* (Cox & Cox, 1997; Cullen, 2005). *Rhododendron ferrugineum* and *R. hirsutum* form a separate group in phylogenetic trees (Khan *et al.*, 2021; Xia *et al.*, 2022) and have a fixed position in the subsection (Nachychnko, Sosnovsky & Bräuchler, 2022). Species of the subsection *Rhododendron* are morphologically similar (Fig. 1), but *R. hirsutum* can be easily distinguished from *R. ferrugineum* and *R. myrtifolium* by its ciliate and less densely lepidote leaves (Popova, 1972). *Rhododendron myrtifolium* differs from *R. ferrugineum* in quantitative characteristics. The former species is no more than 20–40 cm high and leaves are no more than 2 cm long (Komendar, 1996; Danilik, 2009), whereas *R. ferrugineum* is 60–80 cm high in the Alps (Aeschmann *et al.*, 2004) and even reaches 120 cm in the Pyrenees (Gómez, 1993) with leaves 2–4 cm long (Popova, 1972; Gómez, 1993). The fruits (capsules) of both species are similar, but *R. myrtifolium* has stalks 8–20 mm long (Anchev, 1982; Danilik, 2009), whereas in *R. ferrugineum* they are only 8 mm (Popova, 1972) or 7–13 mm long (Gómez, 1993) (Supporting Information, Fig. S1).

Rhododendron spp. emerged as early as the end of the Cretaceous, with different diversification rates during the Cenozoic (Khan *et al.*, 2021). Evergreen rhododendrons may have evolved from the subgenus *Rhododendron*, section *Rhododendron* in Europe, probably commencing during the Early Miocene (Xia *et al.*, 2022). This event was connected with the colonization of the Central European Mountains. The divergence of *R. ferrugineum* and *R. hirsutum* has been estimated to have occurred at the end of the Miocene (Xia *et al.*, 2022: fig. 1), during Miocene climate cooling (Zachos *et al.*, 2001: fig. 2). However, the time of divergence for *R. myrtifolium* and *R. ferrugineum* has so far not been specified. Today these two species are allopatric. *Rhododendron ferrugineum*

occurs mostly in the Pyrenees and Alps, with dispersed single localities in the northern Apennines, Sudetes and Dinaric Alps (Jalas & Suominen, 1976; Căprar *et al.*, 2014; Malicki *et al.*, 2019), whereas *R. myrtifolium* grows mainly in the Carpathians (Mirek, 2020), with dispersed localities in a few mountain ridges in the Balkans (Apostolova, 2011; Voloshchuk & Shumik, 2012; Nachychnko *et al.*, 2022).

Leaf morphology is important when describing *Rhododendron* spp. (Popova, 1972; Davidian, 1982; Cox & Cox, 1997; Polatschek, 1999; Cullen, 2005; Sosnovsky *et al.*, 2017, 2021). In general leaves, especially in the case of woody species, remain the most important element in their identification and in studies on the taxonomy of closely related species and intraspecific differentiation (e.g. Harper, 1989; Kremer *et al.*, 2002; Wang *et al.*, 2007a; Boratyńska *et al.*, 2014; Marcysiak, 2014; Apostol *et al.*, 2017; Sosnovsky *et al.*, 2017; Jasińska *et al.*, 2021, 2022). Over the last decade, study of leaf characteristics with statistical methods has allowed the detection of intraspecific differentiation of *R. ferrugineum* (Lewandowska *et al.*, 2022), *Zelkova carpinifolia* (Pall.) C. Koch (Jasińska *et al.*, 2015) and *Alnus incana* (L.) Moench (Krause-Michalska & Boratyńska, 2013), and the differences between remnant and fossil species of *Zelkova* Spach in Europe and West Asia to be specified (Jasińska *et al.*, 2021, 2022). Characteristics of fruits, as generative organs, may show different patterns of variation compared to those of vegetative organs and are considered more stable (Stace, 1989; Wang *et al.*, 2007b).

Although phenotypic characteristics could be influenced by local environmental conditions (Wang *et al.*, 2014) which can modify resource capture and allocation abilities (e.g. Groom *et al.*, 2004; Kumar *et al.*, 2021), *R. myrtifolium* and *R. ferrugineum* occur in the subalpine vegetation layer of the mountains of Central Europe, which generally have similar climatic and edaphic conditions (Holtmeier, 2003; Körner,



Figure 1. *Rhododendron* species of the Central European mountains: *Rhododendron myrtifolium* (RM) in the Czarnohora Mountains, East Carpathians (photograph: A. Boratyński), *Rhododendron ferrugineum* (RF) in the Pyrenees (photograph: A. Boratyński) and *Rhododendron hirsutum* (RH) in the Alps (photograph: K. Marcysiak).

2003; Körner, Paulsen & Pelaez-Riedl, 2003; Nagy *et al.*, 2003). Despite climatic similarities between the localities of the analysed species, average climate conditions in the Carpathians are generally more continental than those in the Alps and Pyrenees, and the Pyrenees are under the influence of the Atlantic climate (Rivas-Martínez, Peñas & Díaz González, 2004).

Lewandowska *et al.* (2022) recently studied morphological variability in *R. ferrugineum* using leaf and capsule characteristics, pointing out the usefulness of this set for distinguishing populations at the intraspecific level and showing that geographical variation exists among populations in the Pyrenees, Alps and Sudetes. This pattern appears to be consistent with their genetic differentiation (Wolf *et al.*, 2004; Charrier *et al.*, 2014; Suchan, Malicki & Ronikier, 2019). Interspecific variation in *R. myrtifolium* in the Czornohora Mountains indicates a tendency in the species to an increase in leaf xeromorphy with increasing elevation and decreasing water availability (Sosnovsky *et al.*, 2021). Potential intraspecific differentiation relating to geography in *R. myrtifolium* has not been studied yet, but it could be expected, as the geographical range of *R. myrtifolium* has several disjunctions (Boratyński *et al.*, 2006; Mirek, 2020).

Phenotypic differences between *R. myrtifolium* and *R. ferrugineum* have not been investigated. Therefore, in the present study we aimed to define interspecific differences between these two species across their geographical ranges. We expect that the current disjunctive distribution of the studied species should be reflected in interspecific differentiation at the level of their leaf and capsule morphology. Our goal was to verify the degree to which the set of characteristics of leaves and capsules that are informative in the taxonomy of the genus (Davidian, 1982; Chamberlain *et al.*, 1996; Cullen, 2005) allow for distinction between these species.

MATERIAL AND METHODS

PLANT MATERIALS AND MEASUREMENT PROCEDURES

Natural populations of *R. myrtifolium* were sampled in 11 localities, three from the southern Carpathians and eight from the eastern Carpathians (Fig. 2). Data on *R. ferrugineum* came from a study by Lewandowska *et al.* (2022: supplementary material, tables S1 and S2) and included 13 populations of *R. ferrugineum*, five from the Pyrenees, seven from the Alps and one from the Sudetes. Most populations were represented by 26–30 individuals that were separated in the field by *c.* 20 m. This distance provides sufficient spatial isolation, difficult to overcome by vegetatively expanding specimens, even

over several thousand years (Pornon & Escaravage, 1999). Only two small populations of *R. myrtifolium* were represented by 22–23 individuals, and two populations of *R. ferrugineum* were represented by 11 and 20 individuals (Table 1). Each individual was represented by ten 2-year-old leaves that were taken from the central parts of well-insolated shoots. For two populations of *R. myrtifolium* and nine of *R. ferrugineum*, one ripe and uninjured capsule was taken from each of the same individual plants. As these taxa bloom from May to July (in the Iberian Peninsula to August) (Garcke, 1972; Davidian, 1982; Gómez, 1993; Barbarich, 1987), the collections were carried out in September, ensuring that well-ripened capsules were obtained. The materials were dried immediately after sampling; the leaves were cured with a herbarium press, and then stored under herbarium conditions; the capsules were dried in the press without considerable pressure and stored in separate envelopes in herbarium conditions. In total, 3100 leaves from 311 individuals and 60 capsules of *R. myrtifolium* and 3465 leaves from 354 individuals and 252 capsules of *R. ferrugineum* were measured, statistically analysed and compared. Voucher specimens were deposited at the herbarium of the Institute of Dendrology, Kórnik, Poland (KOR) (Table 1). Dry plant material was scanned, and measurements were then performed on the scanned images with the implementation of DIGISHAPE software (Moraczewski, 2005).

We analysed 14 leaf characteristics and 12 capsule characteristics (Supporting Information, Fig. S2), as described by Lewandowska *et al.* (2022: supplementary material, fig. S1A, B). For the leaf characteristics, we used the perimeter of the leaf blade (LP), the length of the blade (LL) and of the petiole (PeL), and width of the leaf blade measured at half of its length (W), at quarter of its length ($W^{\frac{1}{4}}$) and at three-quarters of its length ($W^{\frac{3}{4}}$). Angles of the apex (AA) and the base (BA) of the leaf blade were also measured. Based on these eight measured characteristics, six ratios were calculated (Table 2). For the capsules, the length of stalk (SL) and dimensions of one capsule valve were measured: its perimeter (CP), length (CL), width at half of its length (CW), at quarter of its length ($CW^{\frac{1}{4}}$) and at three-quarters of its length ($CW^{\frac{3}{4}}$), and the angle of the apex. These seven measured characteristics were used to calculate the five ratios (Table 2).

STATISTICAL ANALYSES

Before the mathematical analyses, the statistical distribution of each character was tested for data unimodality and symmetry, using the Shapiro–Wilk test (Shapiro & Wilk, 1965). The variance

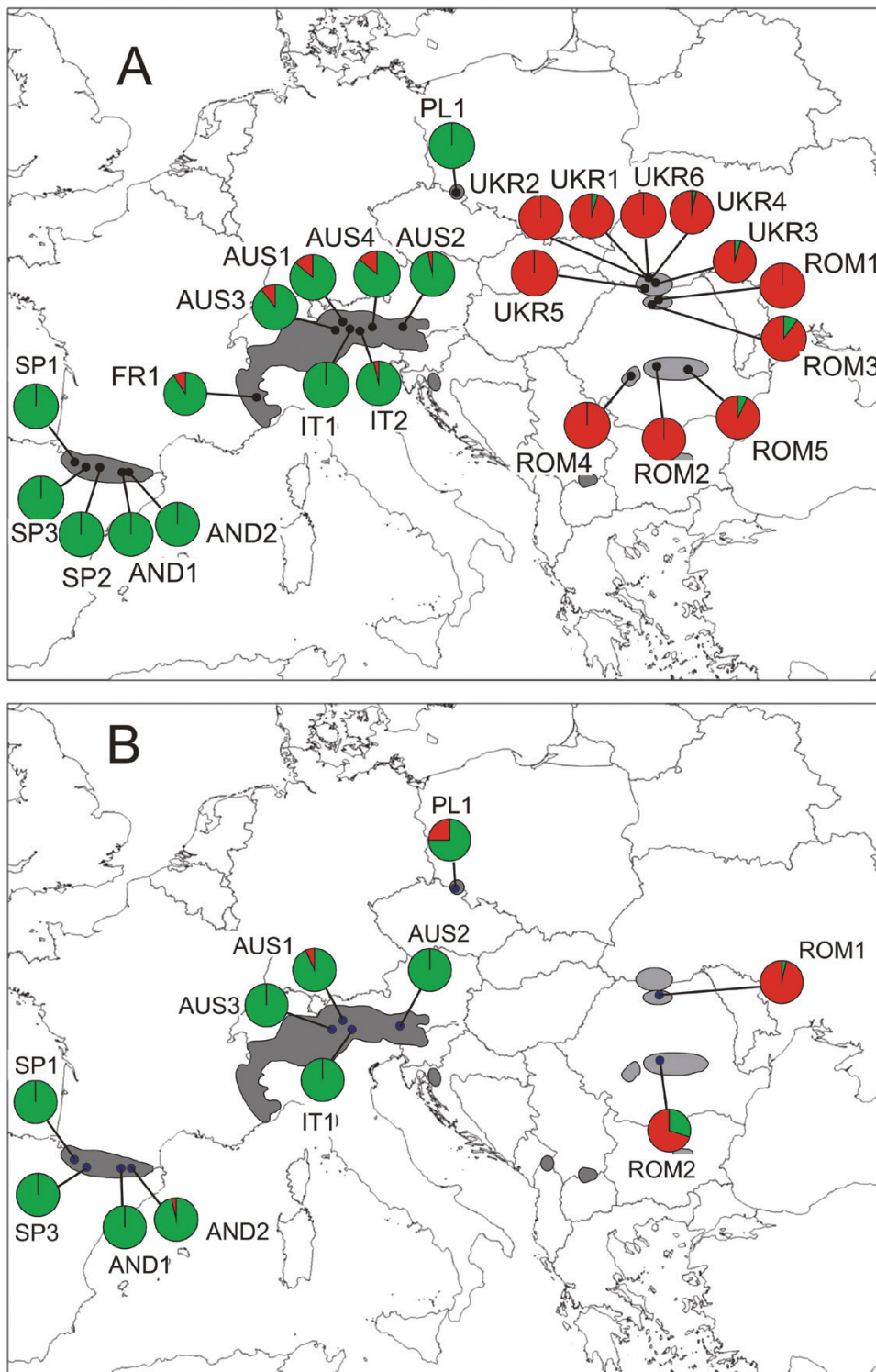


Figure 2. Geographical distribution of the investigated populations of *Rhododendron myrtifolium* (red) and *R. ferrugineum* (green), with percentages of individuals from particular species included in each population (population acronyms as in Table 1); A, all populations (leaves sampled); B, populations from which capsules were collected.

Table 1. Populations of *Rhododendron myrtifolium* and *R. ferrugineum* analysed; for explanation, see Figure 2.

Species	Population acronym	Locality	Voucher no.	Latitude (°)	Longitude (°)	Altitude (m)	Number of individuals	Capsules*	
								Leaves	Capsules
<i>R. myrtifolium</i>	ROM1	E. Carpathians, Romania, Rodna Mts	KOR 55983	47.617	24.700	1750	30	30	30
	ROM2	S. Carpathians, Romania, Negoiu Mt.	KOR 55996	45.617	24.550	2000	30	30	30
	ROM3	E. Carpathians, Romania, Rodna Mts	KOR 55997	47.596	24.638	2200	30	–	–
	ROM4	S. Carpathians, Romania, Parâng Mts	KOR 55998	45.366	23.519	2350	30	–	–
	ROM5	S. Carpathians, Romania, Bucegi Mts	KOR 55999; KOR 17356	45.407	25.485	2050	30	–	–
	UKR1	E. Carpathians, Ukraine, Czarnohora Mts	KOR 56000; KOR 40045	48.133	24.533	1550	22	–	–
	UKR2	E. Carpathians, Ukraine, Czarnohora Mts	KOR 56001; KOR 40065	48.132	24.533	1500	23	–	–
	UKR3	E. Carpathians, Ukraine, Czarnohora Mts	KOR 56002; KOR 40061	48.117	24.567	1900	30	–	–
	UKR4	E. Carpathians, Ukraine, Czarnohora Mts	KOR 56003	48.150	24.517	1750	30	–	–
	UKR5	E. Carpathians, Ukraine, Maramuresh Mts	KOR 56004	47.967	24.483	1750	30	–	–
	UKR6	E. Carpathians, Ukraine, Czarnohora Mts	KOR 56005	48.132	24.532	1620	26	–	–
	AND1	Pyrenees, Andorra, Puig Alt de la Capa	KOR 55984; KOR 32816	42.583	1.450	1700	30	30	30
	AND2	Pyrenees, Andorra, Estany d'Engolasters	KOR 55985	42.517	1.550	1650	30	30	30
	SP1	Pyrenees, Spain, Puerto de Formigal	KOR 55992	42.783	-0.350	1850	29	30	30
	SP2	Pyrenees, Spain, above Lago de Bardamina	KOR 55993	42.667	0.550	2300	30	–	–
	SP3	Pyrenees, Spain, Ordesa	KOR 55994; KOR 25479	42.633	-0.017	1900	30	30	30
IT1	Alps, Italy, Penser Joch	KOR 55990	46.850	11.383	1900	26	30	30	
IT2	Alps, Italy, Königsangspitze	KOR 55991	46.706	11.577	2260	28	–	–	
AUS1	Alps, Austria, Obergurgl–Hochgurgl	KOR 55986	46.880	11.041	2040	30	30	30	
AUS2	Alps, Austria, Spittal–Goldeck	KOR 55987	46.757	13.462	2070	30	30	30	
AUS3	Alps, Austria, Vent–Kreuz Kögl	KOR 55988	46.842	10.914	2100	30	30	30	
AUS4	Alps, Austria, Thurmteler Kögl	KOR 55989	46.773	12.389	2340	30	–	–	
FR1	Alps, France, Col de Vars	KOR 55995	44.546	6.689	2250	11	–	–	
PL1	Sudetes, Poland, Karkonosze		50.740	15.78	1170	20	12	12	

* – no capsules were collected from the population.

Table 2. Mean (M), standard deviation (SD) and variation coefficient (V) for characteristics of *Rhododendron myrtifolium* and *R. ferrugineum* leaves and capsules, with ANOVA results.

Character	Code	Species		ANOVA			
		<i>R. myrtifolium</i>		<i>R. ferrugineum</i>			
		M ± SD	V	M ± SD	V		
Leaf blade perimeter (mm)	LP	37.80 ± 4.27	11.29	53.42 ± 8.91	16.68	78.72	0.000
Leaf blade length (mm)	LL	15.67 ± 1.95	12.42	22.84 ± 4.05	17.75	81.76	0.000
Petiole length (mm)	PeL	2.60 ± 0.42	16.13	4.04 ± 0.75	18.67	59.62	0.000
Leaf blade width at its half-length (mm)	W	6.52 ± 0.68	10.40	8.25 ± 1.28	15.54	41.55	0.000
Leaf blade width at its quarter length (mm)	W¼	5.59 ± 0.59	10.51	6.43 ± 0.89	13.83	21.94	0.000
Leaf blade width at its three-quarter length (mm)	W¾	4.96 ± 0.56	11.26	6.54 ± 1.07	16.37	47.11	0.000
Leaf blade apex angle (°)	AA	119.67 ± 8.32	6.95	92.01 ± 10.62	11.54	107.95	0.000
Leaf blade base angle (°)	BA	106.13 ± 11.99	11.30	95.42 ± 11.86	12.43	23.47	0.000
Ratio of leaf blade length/width at ½	LL/W	2.41 ± 0.24	10.01	2.79 ± 0.34	12.24	33.84	0.000
Ratio of leaf blade perimeter/length	LP/LL	2.42 ± 0.04	1.84	2.35 ± 0.05	1.94	39.41	0.000
Ratio of leaf blade length/petiole length	LL/PeL	6.38 ± 1.03	16.13	5.87 ± 1.01	17.13	17.70	0.000
Ratio of leaf blade width at ¼/width at ¾	W¼/W¾	1.14 ± 0.09	8.18	0.99 ± 0.09	8.98	36.10	0.000
Ratio of leaf apex angle/leaf base angle	AA/BA	1.15 ± 0.13	11.03	0.98 ± 0.11	11.46	38.52	0.000
Ratio of leaf blade width at ¼/width at ½	W¼/W	0.86 ± 0.03	3.56	0.78 ± 0.04	4.73	55.55	0.000
Capsule valve perimeter (mm)	CP	13.53 ± 1.31	9.66	14.22 ± 1.83	12.91	14.87	0.000
Capsule valve length (mm)	CL	5.62 ± 0.58	10.29	6.03 ± 0.83	13.71	15.22	0.000
Stalk length (mm)	SL	21.50 ± 3.74	17.41	13.36 ± 2.66	19.93	63.14	0.000
Capsule valve width at its half-length (mm)	CW	1.85 ± 0.22	11.92	1.67 ± 0.20	11.90	7.50	0.000
Capsule valve width at its quarter length (mm)	CW¼	1.47 ± 0.18	12.19	1.39 ± 0.16	11.57	4.76	0.000
Capsule valve width at its three-quarter length (mm)	CW¾	1.80 ± 0.21	11.52	1.61 ± 0.22	13.54	11.27	0.000
Capsule valve apex angle (°)	CA	102.97 ± 10.40	10.10	98.19 ± 11.84	12.05	2.19	0.019
Ratio of capsule valve length/width at ½	CL/CW	3.06 ± 0.31	10.26	3.64 ± 0.47	12.99	19.99	0.000
Ratio of capsule valve perimeter/length	CP/CL	2.41 ± 0.05	2.04	2.36 ± 0.05	2.10	9.51	0.000
Ratio of capsule valve length/stalk length	CL/SL	0.27 ± 0.05	19.07	0.47 ± 0.10	22.22	50.55	0.000
Ratio of capsule valve width at ¼/width at ¾	CW¼/CW¾	0.82 ± 0.08	9.88	0.87 ± 0.10	11.53	5.91	0.000
Ratio of capsule valve width at ¼/width at ½	CW¼/CW	0.79 ± 0.06	7.29	0.84 ± 0.07	8.03	4.16	0.000

F, results of F-test; P, probability.

homoscedasticities of the characteristics were assessed using the Brown–Forsythe test, to ensure the adequacy of the parametric statistical tests (Zar, 1999; Sokal & Rohlf, 2003). Interactions between the characteristics were then verified using Pearson's correlation coefficient (Garren, 1998) to eliminate possible multicollinearity (Zar, 1999; Sokal & Rohlf, 2003). Before the multivariate analyses, data were standardized using STATISTICA 12 PL software (StatSoft Inc., 2014), to avoid the possible influence of data heterogeneity on the results. Differences between the mean values of characteristics for *R. myrtifolium* and *R. ferrugineum* were tested using a one-way ANOVA and Tukey's honest significance test (Zar, 1999; Sokal & Rohlf, 2003).

Similarities and dissimilarities between the species were evaluated using an analysis of discrimination analysis (DA), principal component analysis (PCA) and agglomeration after Ward's method on Euclidean distances (Sokal & Rohlf, 2003). In the DA and agglomeration analyses, we used proportions and characteristics that were not used in the proportion calculation for leaf: AA, BA, LL/W, LP/LL, LL/PeL, $W_{1/4}/W_{3/4}$ and $W_{1/4}/W$ and for capsule SL, CL/CW, CP/CL, $CW_{1/4}/CW_{3/4}$ and $CW_{1/4}/CW$. Leaf and capsule data were analysed separately. For PCA, we used entire sets of leaf and capsule characteristics.

The possible presence of individuals of both species in the populations was verified with a K-means cluster analysis (K-MCA), which indicates the percentages of individuals correctly classified as *R. myrtifolium* and/or *R. ferrugineum* in every population of the other species (Sneath & Sokal, 1973). Mathematical calculations were performed using STATISTICA 12 PL software for Windows (StatSoft Inc., 2014).

BIOCLIMATIC DATA

The possible relationship of leaf and capsule characteristics to the bioclimatic factors of sampled geographical positions were verified using analysis of regression, via JMP 13 software (SAS Institute Inc.). The climatic data (Supporting Information, Table S1) were retrieved from the WorldClim (WC) database (<http://worldclim.org/>; Fick & Hijmans, 2017), at a resolution of 30 arc-seconds, i.e. c. 1 km in the area of Central Europe.

RESULTS

BIOCLIMATIC FEATURES OF THE SAMPLED POPULATIONS

At the localities of the studied populations, the continental character of the Carpathian climate was manifested by lower temperatures, a lower minimum temperature of the coldest month, -11.54 °C in the

Carpathians compared to -8.18 °C in localities of *R. ferrugineum* (Pyrenees, Alps, Sudetes), and a lower mean temperature of the driest and coldest quarter of the year, -4.80 and -7.30 °C, respectively, vs. -0.09 and -4.42 °C. Annual precipitation in the driest and coldest months was also lower in localities of *R. myrtifolium* than of *R. ferrugineum* (171–180 vs. 220–234 mm, respectively) (Supporting Information, Table S1).

Temperature seasonality (BIO4), max temperature of warmest month (BIO6), mean temperature of coldest quarter (BIO11) and precipitation seasonality (BIO15) generally had the greatest influence on leaf and capsule characteristics (Supporting Information, Table S2). The closest relationships of particular characteristics and bioclimatic factors revealed gradual changes of the latter between populations of *R. myrtifolium* and *R. ferrugineum*, and at the same time indicated differences between the species (Fig. S3).

VARIATION AND CORRELATION OF CHARACTERISTICS

The leaf and capsule characteristics had unimodal and normal or close to normal frequency distributions and homoscedastic variances in most of the populations examined. This allowed the use of multivariate analyses and parametric tests.

The most variable leaf characteristic was petiole length (PeL), with values of V (the variation coefficient) being 16.13 and 18.67% in *R. myrtifolium* and *R. ferrugineum*, respectively. The least variable characteristic was the leaf apex angle (AA), with V values of 6.95% in *R. myrtifolium* and 11.54% in *R. ferrugineum* (Table 2). The V values of the proportions were generally lower than those of the measured characteristics (Table 2).

Leaf dimensional characteristics were significantly and positively correlated to each other ($P < 0.01$), whereas leaf blade apex (AA) and leaf blade base (BA) angles were negatively correlated with all the other measured leaf characteristics for both *R. myrtifolium* and *R. ferrugineum* (Supporting Information, Table S3; Lewandowska *et al.*, 2022: table S2). The closest correlations were found between leaf blade perimeter (LP) and leaf blade length (LL), for both species. From the pairs of correlating characteristics ($r \geq |0.95|$), LP and the leaf blade width measured at half its length (W) were excluded from the multivariable analyses as being redundant.

The capsule characteristics were generally less variable than those of the leaves, with the exception of stalk length (SL), with V values of 17.41% in *R. myrtifolium* and 19.93% in *R. ferrugineum* (Table 2). The measured capsule characteristics were positively and significantly correlated ($P < 0.01$). The only exception

was capsule valve angle (CA), which was negatively correlated with most of the other characteristics in both species. We excluded capsule valve perimeter (CP) from the analyses as being redundant, as it was correlated with capsule valve length (CL) (Supporting Information, Table S3).

DIFFERENCES BETWEEN *R. MYRTIFOLIUM* AND *R. FERRUGINEUM*

Significant differences ($P \leq 0.01$) were present between *R. myrtifolium* and *R. ferrugineum* for all of the leaf and capsule characteristics tested using ANOVA (Table 2). Tukey's test found that the largest number of leaves and capsule characteristics differed significantly between populations of these two species. At the same time, Tukey's test revealed some interspecific differentiation (Supporting Information, Table S5). The Pyrenean populations of *R. ferrugineum* displayed a greater number of significantly different leaf characteristics ($P \leq 0.05$) than other populations of *R. ferrugineum*. Alpine populations of *R. ferrugineum* had fewer leaf characteristics that differed from the *R. myrtifolium* populations. Additionally, the *R. ferrugineum* population AUS4, from the East Alps, was less different to populations of *R. myrtifolium* than to the other tested populations representing *R. ferrugineum* (Table S5A). The populations of both species were less different with regard to capsule characteristics. However, in the Pyrenees, *R. ferrugineum* populations had the highest number of differences compared to *R. myrtifolium*, with more differences in characteristics than populations from the Alps and Sudetes (Table S5B).

DA based on leaf characteristics indicated that species differentiation was most highly influenced by the ratio of leaf blade length to petiole length (LL/PeL), the apex angle of the leaf blade (AA), and the ratio of the blade width at a quarter of the length of the leaf blade to its width at three-quarters of the leaf blade length ($W_{1/4}/W_{3/4}$), with Wilks' partial lambda values of 0.60, 0.64 and 0.73, respectively. For capsule characteristics, the length of the capsule stalk (SL) and the ratio of capsule length to the width of the capsule valve measured at half of its length (CL/CW) influenced the differentiation between species at the highest degree, with Wilks' partial lambda values of 0.36 and 0.72, respectively.

Population dispersion on the space between the two first discrimination variables based on leaf characteristics discriminated between the species well, accounting for 81.4% of the total variation (Fig. 3A). The dispersion of *R. myrtifolium* and *R. ferrugineum* populations in the space between the two first discrimination variables for capsule characteristics also indicated a clear separation between the species,

accounting for 91.3% of the total variation (Fig. 3B). However, in both cases, the dispersion of individuals from *R. myrtifolium* partly overlapped with individuals of *R. ferrugineum* (Supporting Information, Fig. S4).

PCA on the leaf characteristics clearly distinguished between populations of *R. myrtifolium* and those of *R. ferrugineum*, which formed two separate groups in the space between the two first components and was responsible for 88.6% of the total variation (Fig. 3C), this being determined mainly by LP, LL and AA (Supporting Information, Fig. S5). Similarly, PCA on the capsule characteristics distinguished between populations of the two species (Fig. 3D). The differences between individuals, however, were not so clear. Individuals of both species were partly intermixed on the dispersion diagram in the space between the two first PCA variables. These particular individuals fell within the 95% confidence intervals between species with regard to leaf and capsule characteristics (Fig. S6).

The use of Ward's method with leaf and capsule characteristics to test for the agglomeration of *R. myrtifolium* and *R. ferrugineum* populations on the Euclidean distances clearly distinguished between the two species. However, K-MCA found single individuals with characteristics resembling *R. myrtifolium* in populations of *R. ferrugineum*, and vice versa (Fig. 4). Individuals of *R. myrtifolium* were present in *R. ferrugineum* populations in the Alps, and, in the case of capsule characteristics, also in the Sudetes, but they were absent in the Pyrenees. Single individuals of *R. ferrugineum* were present in both the Eastern and Southern Carpathian populations of *R. myrtifolium* (Fig. 2).

DISCUSSION

TAXONOMIC RELATIONSHIPS OF *R. MYRTIFOLIUM* AND *R. FERRUGINEUM*

Our study represents the first comparison between populations of *R. myrtifolium* and *R. ferrugineum* over nearly the entire geographical range of the two species on the basis of leaf and capsule morphometric characteristics. The differences between these two species were recognized as mostly quantitative (Braun-Blanquet, 1926; Popova, 1972; Davidian, 1982). We detected the most evident differences between *R. myrtifolium* and *R. ferrugineum* from analyses of the morphometric characteristics of their leaves (LP, LL, PeL, W, $W_{1/4}$, $W_{3/4}$, AA and BA; Table 2). Although the proportional ratios between these features were significantly distinct between the two species ($P < 0.01$), relatively smaller differences compared to the directly measured leaf characteristics were observed (Table 2). This indicated a rather common pattern of leaf form

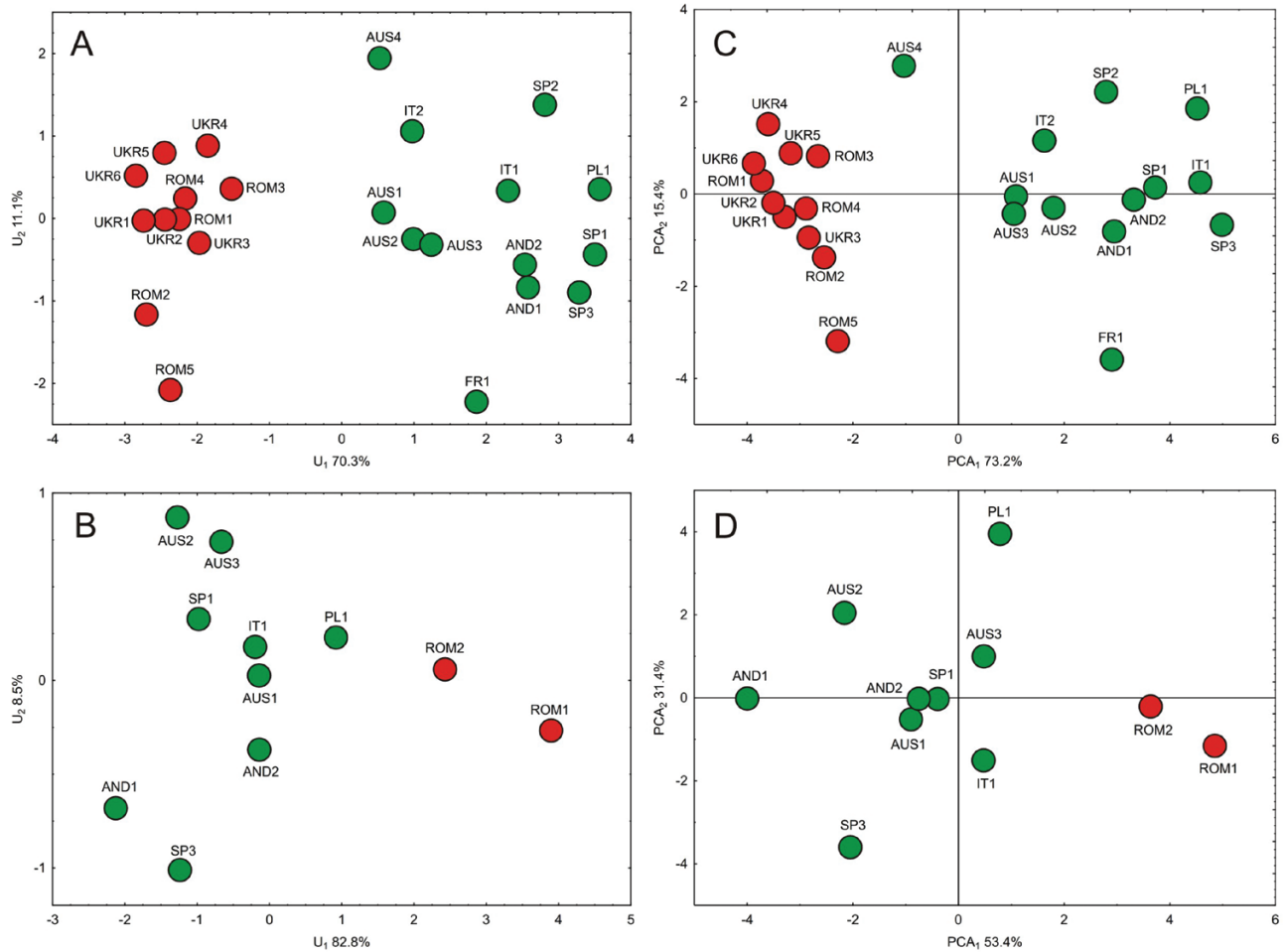


Figure 3. Dispersion of *Rhododendron myrtifolium* populations (red dots) and *R. ferrugineum* populations (green dots) in the space between the two first discrimination variables, on the basis of leaf (A) and capsule (B) characteristics; relationships between *R. myrtifolium* and *R. ferrugineum* populations using principal component analysis (PCA) on leaf (C) and capsule (D) characteristics.

in *R. myrtifolium* and *R. ferrugineum*, despite the dimensional differences of these organs in the two species. Similarly, capsule characteristics allowed for populations of *R. myrtifolium* to be distinguished from *R. ferrugineum*. However, the ranges of variation for each leaf and capsule characteristic overlapped between the two species, and consequently, not every individual could be identified using the characteristics studied. Despite this, differences in the morphological construction patterns of leaf and capsule detected in the study confirm the taxonomic status of *R. myrtifolium* and *R. ferrugineum*.

PRESUMED EFFECTS OF ENVIRONMENTAL CONDITIONS ON OBSERVED VARIABILITY

Leaves have specific phenotypic and functional characteristics that are crucial for plant growth and survival (Reich, Walters & Ellsworth, 1997; Wright

et al., 2004; Diaz *et al.*, 2016). Differences in the environmental conditions of the studied localities were linked to leaf shape differences in *Rhododendron mucronulatum* Turcz. in East Asia (Koksheeva *et al.*, 2017), and in European *R. myrtifolium*, diverse site conditions in the Eastern Carpathians have explained inconspicuous differences in the leaf shapes of individual plants (Sosnovsky *et al.*, 2021). The average yearly temperatures of the localities have a slight influence on the apical angles of *R. ferrugineum* leaves (Lewandowska *et al.*, 2022). Conversely, the proportions of dimensional traits in our study were not found to be sensitive to environmental changes at the population level (Marcysiak, 2012a, b; Lewandowska *et al.*, 2022).

From among the bioclimatic factors analysed in the present paper (Supporting Information, Table S1), climate seasonality, especially temperature seasonality, was recognized as being a good predictor

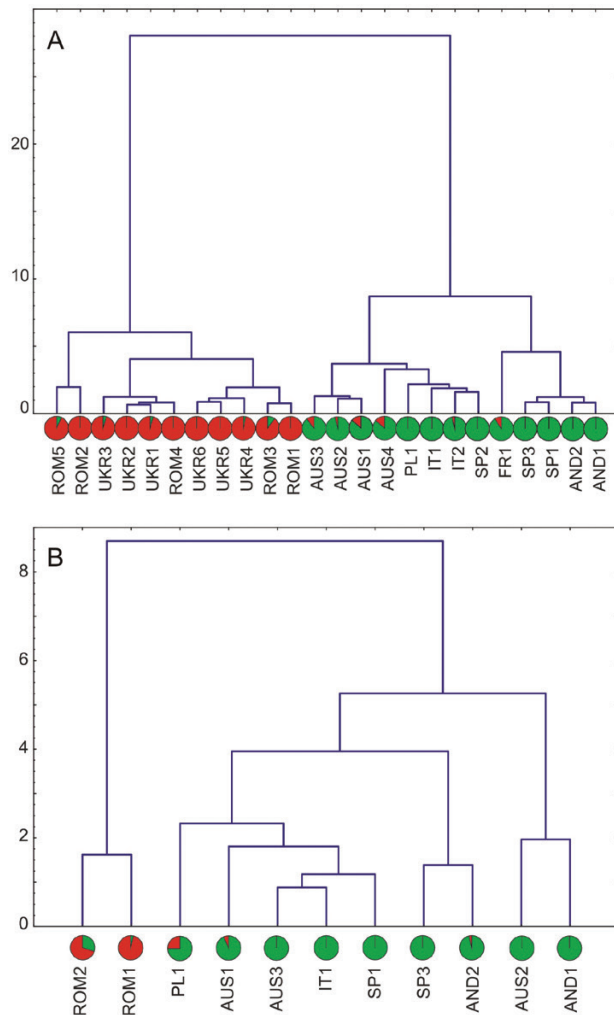


Figure 4. Dendrograms constructed according to Ward's method on the closest Euclidean distances among populations of *Rhododendron myrtifolium* (red) and *R. ferrugineum* (green), using leaf (A) and capsule (B) characteristics.

of species richness in *Rhododendron* in China (Shrestha *et al.*, 2018b), and this factor could also have been decisive for the processes of adaptation and phenotypic differentiation in the mountains of Central Europe.

Different climatic conditions in the Carpathian and Alpine subalpine vegetation belts probably resulted in evolution via adaptation of the ancestor of the remnant species (Thiel-Egenter *et al.*, 2009). Despite *R. myrtifolium* and *R. ferrugineum* predominantly occurring on northern slopes and in micro-terrains formed from the accumulation of snow during winter and more humid conditions during vegetative growth (Boratyński *et al.*, 2006; Gómez *et al.*, 2020), the relatively smaller leaves of *R. myrtifolium*, compared to *R. ferrugineum*, could have

resulted from the evolution of *R. myrtifolium* under harsher environmental conditions. This supports the hypothesis that leaf dimension reduction correlates with the deterioration of climatic conditions over the course of plant evolution (Körner *et al.*, 1989; Wolfe, 1995; Givnish, 2002; Milne, 2004; Iszkuło & Myślicka, 2015). In potential support of this thesis, Sosnovsky *et al.* (2021) observed a tendency for *R. myrtifolium* leaves to exhibit more xeromorphic structures correlated with generally harsher site and climatic conditions within localities at different elevations and exposures in the East Carpathians.

POSSIBLE MIGRATION HISTORIES OF *R. MYRTIFOLIUM* AND *R. FERRUGINEUM*

Rhododendron ferrugineum and *R. hirsutum* diverged during the Pliocene (Xia *et al.*, 2022). Considering the lower level of phenotypic differences between *R. myrtifolium* and *R. ferrugineum* than between them and *R. hirsutum* that is currently observed (Sosnovsky *et al.*, 2017), we can conclude that the divergence between *R. myrtifolium* and *R. ferrugineum* probably took place no earlier than the end of Pliocene. However, extensive examination involving demographic reconstruction based on molecular approaches may shed more light on this hypothesis.

This event was potentially linked to the Pleistocene climate oscillations, which may have caused isolation/contact periods between the Carpathian, Alpine and Pyrenean populations. However, fossil data for *Rhododendron* are scarce, impeding accurate reconstructions of the biogeographical history of the genus (Collinson & Crane, 1978; Palamarev *et al.*, 2005; Denk *et al.*, 2011).

Contact between *R. myrtifolium* and *R. ferrugineum* populations during the Pleistocene may explain the current presence of single individuals with leaves and capsules of *R. myrtifolium* in several populations of *R. ferrugineum* in the Alps, and vice versa (Fig. 2). These could indicate occurrence of mixed populations of the two species in the Alps and Carpathians or individuals of hybrid origin in the populations of every species with intermediate leaf and capsule characteristics. *Rhododendron* spp. frequently hybridize (e.g. Milne *et al.*, 1999; Milne, Terzioglu & Abbott, 2003; Milne & Abbott, 2008; Bruni *et al.*, 2016), but hybridization between *R. myrtifolium* and *R. ferrugineum* has not been described until now. Populations of *R. ferrugineum* from the Pyrenees did not demonstrate evidence for the impact of *R. myrtifolium* on leaf and capsule morphology.

Rhododendron myrtifolium and *R. ferrugineum* can be treated as another example of a recognizable phenomenon of Carpathian (Balkan)–Alpine vicariance. To date, several such pairs of vicariant species have

been described, including: *Salix kitaibeliana* Willd.–*Salix retusa* L. (Jalas & Suominen, 1976; Kosiński, Boratyński & Hilpold, 2017), *Salix silesiaca* Willd.–*Salix appendiculata* Vill. (Jalas & Suominen, 1976), *Doronicum stiriacum* (Vill.) Dalla Torre (Carpathians and Eastern Alps)–*Doronicum clusii* (All.) Tausch (Central and Western Alps) (Pachschwöll *et al.*, 2015), and *Pilosella alpicola* F.W.Schultz & Sch.Bip. (Alps)–*Pilosella rhodopea* (Griseb.) Szeląg (Carpathians and Rhodopes) (Šingliarová, Šuvada & Mráz, 2013).

CONCLUSIONS

Rhododendron myrtifolium and *R. ferrugineum* clearly differ in terms of morphological features. The set of leaf and capsule features used allowed for their differentiation and is therefore suitable for taxonomic studies. Our research shows that the differences between species mainly concern the size of the examined organs. The differences in shape determined by proportions of measured features was not as marked, but was also significant.

We found single individuals with *R. myrtifolium* leaf and capsule morphology in some Alpine and Sudetan *R. ferrugineum* populations, and individuals resembling *R. ferrugineum* in the populations of *R. myrtifolium*. This could indicate contact between these two species during the Pleistocene due to climate oscillations. In our opinion, the slight morphological differences between the studied species indicate their late divergence, possibly in the late Pliocene and/or Pleistocene.

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DATA AVAILABILITY

Mean values and basic statistics on the characteristics of *Rhododendron myrtifolium* populations are available online in [Supporting Information, Tables S6, S7](#). Mean values and basic statistics on the characteristics of *R. ferrugineum* populations are also available at: <https://doi.org/10.12657/denbio.087.001>

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

Figure S1. Scanned leaves (A) and capsules (B) of *Rhododendron myrtifolium* (RM) and *R. ferrugineum* (RF); left column: adaxial (upper) and right column: abaxial (underside) leaf surfaces in every leaf category; scale bar = 1 cm.

Figure S2. Method of measuring characters of leaves (A) and capsules (B); character codes in [Table 2](#).

Figure S3. Examples of statistically significant relationships between values of characteristics of leaf (acronyms as in [Table 2](#)) and average yearly bioclimatic factors (acronyms as in [Table S1](#)); dots indicate positions of populations of *Rhododendron myrtifolium* (red) and *R. ferrugineum* (green).

Figure S4. Dispersion of *Rhododendron myrtifolium* (red dots) and *R. ferrugineum* (green dots) individuals in the space between the two first discrimination variables on the basis of leaf (A) and capsule (B) characteristics; ellipses indicate the 95% confidence interval.

Figure S5. Influence of leaf (A) and capsule (B) characteristics on the dispersion of populations of *Rhododendron myrtifolium* and *R. ferrugineum* ([Fig. 3](#)); see [Table 2](#) for acronyms.

Figure S6. Relationships among individuals of *Rhododendron myrtifolium* (red) and *R. ferrugineum* (green) as revealed in principal component analysis (PCA) on leaf (A) and capsule (B) characteristics; ellipses indicate 95% confidence intervals for each species; influence of leaf (C) and capsule (D) characteristics on the dispersion of *Rhododendron myrtifolium* and *R. ferrugineum* individuals. See [Table 2](#) for acronyms.

Table S1. Bioclimatic data of analysed *Rhododendron myrtifolium* and *R. ferrugineum* populations.

Table S2. Statistically significant relationships between leaf (A) and capsule (B) characteristics and average yearly values of bioclimatic variables.

Table S3. Correlation coefficients of measured leaf characteristics for *Rhododendron myrtifolium*.

Table S4. Correlation coefficients of measured capsule characteristics for *Rhododendron myrtifolium*.

Table S5. Results of Tukey's honest significance test ($P \leq 0.01$ and $P \leq 0.05$) between populations (see [Table 1](#) for codes) for *Rhododendron ferrugineum* and *R. myrtifolium* for leaf (A) and capsule (B) characteristics. See [Table 2](#) for acronyms.

Table S6. Mean (M), minimal (Min) and maximal (Max) values and variation coefficients (V) of analysed characters of *Rhododendron myrtifolium* leaves; population acronyms as in [Table 1](#).

Table S7. Mean (M), minimal (Min), maximal (Max) values and variation coefficients (V) of analysed characters of *Rhododendron myrtifolium* valves of capsules; population acronyms as in [Table 1](#).