

## Pollen and seed morphology of resurrection plants from the genus *Ramonda* (Gesneriaceae): relationship with ploidy level and relevance to their ecology and identification

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**Abstract:** The pollen and seeds of 3 paleoendemic resurrection species, *Ramonda myconi* (L.) Rchb. (Iberian Peninsula), *R. nathaliae* Pančić & Petrović, and *R. serbica* Pančić (Balkan Peninsula), as well as of natural hybrids between the 2 last species, have been analyzed using light and scanning electron microscopy. Their general structural differences, taxonomic and phylogenetic significance, pollen viability, and seed germination capacity, as well as the correlation of pollen and seed characteristics and plant ploidy levels, have been studied. The pollen grains are small (*R. myconi*, *R. nathaliae*) to medium (*R. serbica*) in size, ranging from 10 to 28 µm, and 3-colporate, isopolar monads with microreticulate-perforate exine. Seeds are small, 309 to 1000 µm long and 80 to 425 µm wide, elongated, with a reticulate surface and auriculate ornamentation. Micromorphologies of the pollen exine ornamentation and seed surface revealed significant differences among the 3 species. Pollen from hybrid individuals was heterogeneous in size and morphology, and the germination of their tiny seeds was very low. A strong correlation was found between pollen size, DNA content, and chromosome number. The environmental influence on pollen and seeds of 3 species and especially of the *R. nathaliae* population growing on serpentine is also discussed.

**Key words:** *Ramonda*, Gesneriaceae, pollen, seeds, hybrids, cytometry

### 1. Introduction

The family Gesneriaceae is mainly distributed in the tropics and subtropics of both the Old and New Worlds with only a small number of species extending to temperate regions. Among these are 3 *Ramonda* Rich. species. *Ramonda myconi* (L.) Rchb., inhabits the Iberian Peninsula, while *R. serbica* Pančić and *R. nathaliae* Pančić & Petrović are found in the Balkan Peninsula. All 3 species are long-lived and slow-growing evergreen rosette hemicryptophytes. As relicts, paleoendemic species that are remnants of the Tertiary flora in Europe, they show a fragmented distribution, occupying similar refugia-type habitats in gorges, canyons, ravines, and steep mountain slopes. Usually in the summer and early autumn when rainfall is often very limited and temperatures are still high, they are subjected to drought stress and they go into an anabiotic state, as do other poikilohydric or resurrection plants.

The origin of European representatives of Gesneriaceae and the relationships of the 3 *Ramonda* species are still

open questions. Although modern molecular phylogenies have shown a close relationship between the European genera of Gesneriaceae and their rather basal position (Weber, 2004; Möller et al., 2009; Wang et al., 2010), none of these investigations have included all 5 species.

The distribution of *Ramonda serbica* today extends from NE, SW, and SE Serbia and NW Bulgaria, through SE Montenegro and Albania, to W Macedonia and NW Greece. *Ramonda nathaliae* is restricted to Central Macedonia and North Greece with several localities in SE Serbia (Košanin, 1921; Stevanović et al., 1986a, 1986b, 1991). The 2 species cooccur only in SE Serbia, establishing a sympatric zone with mixed syntopic populations (Stevanović et al., 1986b). On the other side of Europe, *R. myconi* survives in limestone gorges and humid mountain valleys of the Pyrenees (Pico and Riba, 2002; Dubreuil et al., 2008). They all prefer limestone rocks, but *R. nathaliae* can also be found on ophiolitic bedrock (Košanin, 1921; Stevanović and Stevanović, 1985).

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Sympatric populations of *Ramonda nathaliae* and *R. serbica* in the localities Radovanski Kamen and Oblik were discovered and described more than 20 years ago (Stevanović et al., 1986b). However, only recently has cytogenetic analysis of these populations shown that among the diploid individuals of *R. nathaliae* ( $2n = 48$ ) and hexaploid *R. serbica* ( $2n = 144$ ), there are also tetraploid hybrids with  $2n = 96$  that, morphologically, appear to be more similar to *R. serbica* (Siljak-Yakovlev et al., 2008).

Although pollen and seeds of some representatives of the family Gesneriaceae have been investigated in the past (Erdtman, 1952; Melhelm and Mauro, 1973; Williams, 1978; Fritze and Williams, 1988; Sontag and Weber, 1998; Kvist and Skog, 1992; Luegmayr, 1993a, 1993b; Schlag-Eidler and Kiehn, 2001; Fournery et al., 2010; Fuchs et al., 2011), only general descriptions of *Ramonda* pollen grains and seeds can be found (Beaufort-Murphy, 1983; Weber, 2004). Thus, the results presented in the present paper constitute the first comparative, detailed investigation of the pollen and seed morphology of these 3 *Ramonda* species.

Many researchers have found positive correlations between genome size and some phenotypic plant traits, such as seed mass, dimensions of different types of cells, or plant height (Beaulieu et al., 2007; Knight and Beaulieu, 2008; Avci et al., 2013). The hypothesis is that species with larger genomes tend to have larger cells and heavier seeds through genome size effect on cell size within seed organs (Beaulieu et al., 2007; Linkies et al., 2010).

The aims of this paper were: 1) to determine the viability and morphology of pollen grains from all 3 *Ramonda* species, and especially from hybrid individuals between *R. nathaliae* and *R. serbica*; 2) to investigate the morphology and germination capacity of their seeds; 3) to determine whether morphological characters of pollen and seeds can provide additional taxonomic and phylogenetic information that would shed light on the relationships between the 3 species; and 4) to explore whether there is a correlation between ploidy level and characteristics of pollen grains and seeds.

## 2. Materials and methods

### 2.1. Plant material sampling

Plant material was collected from 1 population of *Ramonda myconi* from Spain, 3 populations of *R. nathaliae*, 3 populations of *R. serbica*, and 2 sympatric populations situated in SE Serbia (Oblik and Radovanski Kamen) (Table 1). In these last 2 populations, material was gathered from both parental species and hybrid individuals. Voucher specimens are deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac" (BEOU), Faculty of Biology, University of Belgrade, except for *R. myconi*, deposited in the Herbarium of the University of Barcelona (BCN).

### 2.2. Chromosome number and genome size estimation

Chromosome numbers and total DNA amounts were determined as described by Siljak-Yakovlev et al. (2008).

Table 1. List of studied populations of *Ramonda* species.

No.	Species	Locality	Voucher No.
1	<i>R. myconi</i>	Spain: between Gerri de la Sal and Enseu	285 BCN
2	<i>R. nathaliae</i>	Macedonia: gorge of r. Pčinja near Veles	31670 BEOU
3	<i>R. nathaliae</i>	E Serbia: Mt Suva Planina near Divljana	20638 BEOU
4	<i>R. nathaliae</i>	Macedonia: gorge of r. Vardar, near Veles	33186 BEOU
5	<i>R. nathaliae</i>	SE Serbia: Radovanski Kamen	20641 BEOU
6	<i>R. nathaliae</i>	SE Serbia: gorge of r. Nišava, Oblik	20637 BEOU
7	<i>R. serbica</i>	NW Greece: Mt Timfi, gorge of r. Vikos	31674 BEOU
8	<i>R. serbica</i>	E Serbia: gorge of r. Jelašnica, near Niš	10944 BEOU
9	<i>R. serbica</i>	SW Serbia: gorge of r. Godulja	31677 BEOU
10	<i>R. serbica</i>	SE Serbia: Radovanski Kamen	31672 BEOU
11	<i>R. serbica</i>	SE Serbia: gorge of r. Nišava, Oblik	20634 BEOU
12	Hybrids	SE Serbia: Radovanski Kamen	20640 BEOU
13	Hybrids	SE Serbia: gorge of r. Nišava, Oblik	20636 BEOU

In this way, the ploidy level of each studied individual was checked. This was particularly important in sympatric populations, and so genome size was measured for the same 10 hybrid individuals from whom pollen grains and seeds were gathered.

### 2.3. Preparation of pollen for light and scanning electron microscopy

Pollen grains were acetolyzed according to the methods of Erdtman (1952). For light microscopy (LM) studies, measurements were performed on acetolyzed grains mounted for at least 3 weeks in glycerin jelly. The following properties of 50 pollen grains per population were measured: length of polar (P) and equatorial (E) axes, P/E ratio, exine thickness (in equatorial view), distance between the apices of 2 ectocolpi (d), equatorial axis in polar view (D), and apocolpium or polar area index (d/D). All measurements were made under a 40× objective lens. Only well-formed pollen grains were measured. Pollen shape classes were identified based on P/E ratio according to Erdtman's (1943) classification. For scanning electron microscopy (SEM), the acetolyzed pollen grains were mounted on glass cover slips and attached to aluminum stubs. After sputter coating for 100 s at 20 mA (BAL-TEC, SCD 005 Sputter Coater), pollen grains were observed under JEOL JSM-6460LV electron microscope at 25 kV and JEOL JSM-6390 electron microscope at 13 kV. In this way, details of the exine ornamentation were observed, lumen and murus width were measured, and number of lumens in polar and equatorial areas were counted for one population per species and for hybrid individuals. Pollen terminology follows that of Punt et al. (2007) and Hesse et al. (2009).

### 2.4. Estimation of pollen viability

Pollen grains from at least 10 individuals per population were tested for pollen viability using differential staining with Alexander reagent (Alexander, 1969). In sympatric populations this was done separately for parental and hybrid individuals.

### 2.5. Morphology and germination capacity of seeds

Lengths and widths of 50 seeds per population were measured using an LM 5× objective lens. For hybrids only, 50 seeds per individual (10 individuals per population) were measured. For SEM, seeds were coated with gold for 100 s in a sputter-coater and morphological observations were made with a JEOL JSM-6460LV electron microscope at 25 kV. Seed terminology follows that of Barthlott (1981), Beaufort-Murphy (1983), and Weber (2004). The length and width of the 10 widest testa cells and the width of the corresponding murus (at the middle part) were measured for one population per species and for hybrid individuals. Seed weight was obtained for a batch of 100 seeds, with 5 replicates, using an electronic balance and, after recalculation, was presented as the mean weight of

1 seed. Germination capacity of 100 seeds per population was tested in petri dishes with moistened filter paper at a constant temperature of 25 °C for a period of 21 days.

### 2.6. Statistical analysis

Statistical analyses were made using STATISTICA 5.1. All variables were tested for normality by the Shapiro–Wilk W test and came out normally distributed. Nested-design ANOVA was used to test for significant differences between population and species means. A principal components analysis (PCA) was performed for the complete set of data to reveal variation and principal components at different levels (population and species). After this, all data were treated by canonical discriminant analyses (CDAs) in order to check the hypothesis that the analyzed sample was composed of groups differentiated from each other. Finally, the Mahalanobis distance matrix between examined populations of the genus *Ramonda* was used as the basis for an unweighted pair group average (UPGA) cluster analysis performed to interpret relationships between populations and species. Partial correlations were calculated to analyze relationships between investigated characters and P-values were corrected according to Bonferroni adjustment.

## 3. Results

### 3.1. Chromosome number and genome size

The chromosome numbers and genome sizes of 3 *Ramonda* species are presented in Table 2. *Ramonda myconi* and *R. nathaliae* are diploids with  $2n = 48$ , *R. serbica* is a hexaploid with  $2n = 144$ , and all hybrid individuals investigated are tetraploids ( $2n = 96$ ). The 2C DNA and 1Cx values highlight the clear differentiation between the 2 diploid species that is not apparent in chromosome number. The 1Cx value for *R. serbica* was closer to that of the Iberian *R. myconi* than of *R. nathaliae*. The serpentine population of *R. nathaliae* from the gorge of the river Pčinja had the smallest genome size ( $2C = 2.27$  pg) of all the examined populations.

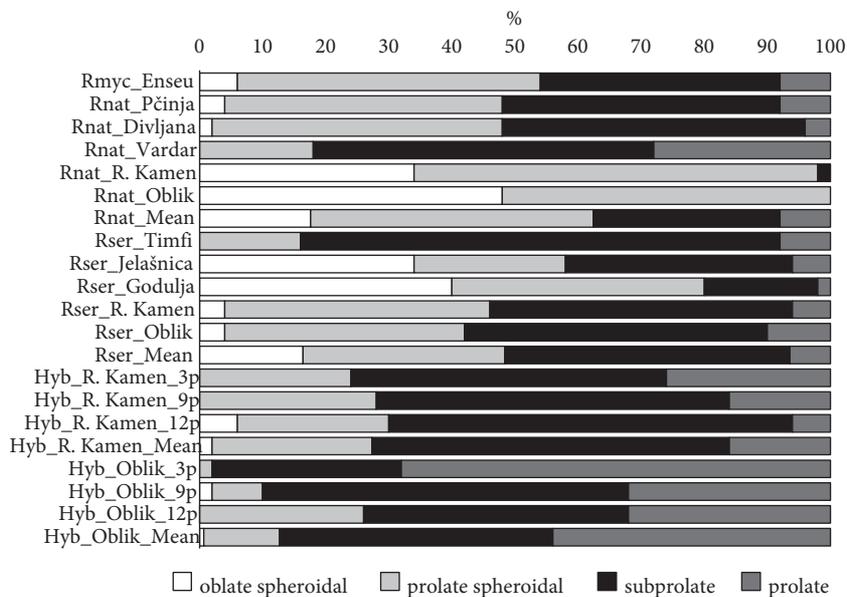
### 3.2. Pollen morphology and viability

The pollen grains of the 3 *Ramonda* species are isopolar monads. Pollen shape is oblate-spheroidal to prolate (Figure 1). Frequencies of forms differ slightly in sympatric populations. Pollen grains are small in *R. myconi* and *R. nathaliae* and medium-sized in *R. serbica* and hybrid individuals (Tables 3 and 4; Figure 2). Pollen grain size of hybrid individuals is very variable. Exine thickness is smallest in *R. nathaliae* and hybrid individuals and thickest and most variable in *R. myconi* (Tables 3 and 4). The thinnest exine was recorded in an *R. nathaliae* population from serpentine. The distances between the apices of the 2 ectocolpi (d) are smallest in *R. myconi* and largest in *R. serbica*. In hybrid individuals, these distances were measured only for grains that are 3-colporate, and they were intermediate between those of *R. nathaliae* and *R. serbica*.

**Table 2.** Chromosome numbers and nuclear DNA contents of 3 *Ramonda* species and hybrid individuals (N: number of plants, <sup>a</sup>: pg = 978 Mbp (Doležel et al., 2003), \*: 2C values and/or chromosome numbers from Siljak-Yakovlev et al. (2008).

No.	Species	Locality	2n	2C in pg and (Mbp) <sup>a</sup>	N	SD	1Cx in pg and (Mbp)
1	<i>R. myconi</i>	Sp: Enseu		2.55 (2493)	5	0.02	1.27 (1246)
2	<i>R. nathaliae</i>	Mac: Pčinja	48	2.27 (2217)	5	0.03	1.13 (1108)
3*	<i>R. nathaliae</i>	Srb: Divljana	48	2.33 (2277)	5	0.05	1.16 (1138)
4	<i>R. nathaliae</i>	Mac: Vardar		2.35 (2295)	5	0.01	1.17 (1147)
5*	<i>R. nathaliae</i>	Srb: R. Kamen	48	2.33 (2279)	5	0.04	1.17 (1139)
6*	<i>R. nathaliae</i>	Srb: Oblik		2.32 (2273)	5	0.02	1.16 (1136)
	<i>R. nathaliae</i>	Mean value		2.32 (2268)			1.16 (1134)
7	<i>R. serbica</i>	Gr: Timfi	144	7.58 (7416)	5	0.17	1.26 (1236)
8*	<i>R. serbica</i>	Srb: Jelašnica	144	7.85 (7678)	5	0.07	1.31 (1280)
9	<i>R. serbica</i>	Srb: Godulja		7.53 (7363)	5	0.53	1.25 (1227)
10	<i>R. serbica</i>	Srb: R. Kamen	144*	7.63 (7460)	5	0.16	1.27 (1243)
11*	<i>R. serbica</i>	Srb: Oblik		8.08 (7900)	5	0.26	1.35 (1317)
	<i>R. serbica</i>	Mean value		7.73 (7563)			1.29 (1261)
12	Hybrids	Srb: R. Kamen	96*	5.20 (5086)	10	0.32	1.30 (1271)
13	Hybrids	Srb: Oblik	96*	5.09 (4979)	10	0.03	1.27 (1245)

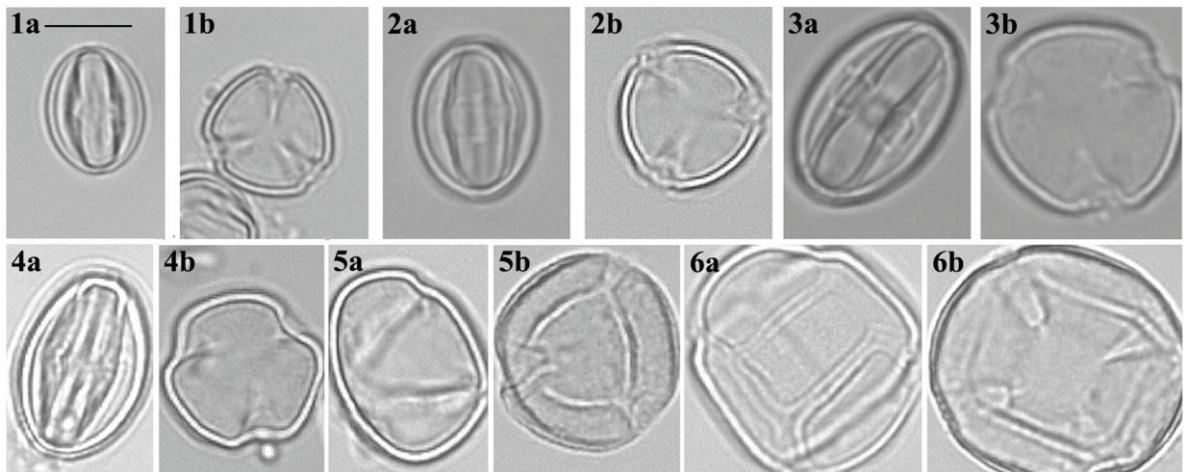
Sp: Spain, Mac: Macedonia, Srb: Serbia, Gr: Greece.



**Figure 1.** Histogram of frequencies of pollen shape categories in *Ramonda*.

**Table 3.** Pollen morphology of 3 *Ramonda* species expressed as mean value  $\pm$  SD (values in  $\mu\text{m}$ , except P/E and d/D).

Species	Locality	P	E	Exine	P/E	d	D	d/D
<i>R. myc</i>	Enseu	14.24 (1.00)	12.57 (0.82)	1.29 (0.37)	1.14 (0.10)	3.61 (0.66)	13.21 (1.08)	0.27 (0.05)
<i>R. nat</i>	Pčinja	15.65 (1.07)	13.65 (0.87)	0.86 (0.23)	1.15 (0.12)	4.55 (1.05)	14.57 (0.90)	0.31 (0.07)
<i>R. nat</i>	Divljana	17.76 (1.15)	15.47 (1.05)	1.16 (0.17)	1.15 (0.09)	4.37 (0.90)	16.76 (0.94)	0.26 (0.06)
<i>R. nat</i>	Vardar	16.47 (1.49)	13.11 (1.02)	0.97 (0.19)	1.26 (0.15)	3.72 (0.70)	14.52 (1.06)	0.26 (0.05)
<i>R. nat</i>	R. Kamen	16.95 (0.69)	16.74 (0.76)	1.13 (0.29)	1.01 (0.05)	3.90 (0.81)	16.85 (0.70)	0.23 (0.05)
<i>R. nat</i>	Oblik	16.90 (0.78)	16.97 (0.71)	1.07 (0.22)	1.00 (0.04)	4.22 (0.68)	17.13 (0.77)	0.25 (0.04)
<i>R. nat</i>	Mean	16.75 (1.27)	15.19 (1.81)	1.04 (0.25)	1.12 (0.14)	4.15 (0.89)	15.97 (1.46)	0.26 (0.06)
<i>R. ser</i>	Timfi	23.09 (1.08)	19.21 (1.03)	1.09 (0.20)	1.21 (0.10)	5.81 (1.37)	19.52 (1.23)	0.30 (0.07)
<i>R. ser</i>	Jelašnica	20.35 (2.08)	18.68 (1.47)	1.02 (0.19)	1.09 (0.13)	5.68 (0.99)	21.06 (2.25)	0.27 (0.05)
<i>R. ser</i>	Godulja	22.00 (2.03)	21.14 (1.33)	1.21 (0.24)	1.05 (0.13)	6.46 (1.43)	22.10 (1.40)	0.29 (0.06)
<i>R. ser</i>	R. Kamen	22.87 (1.33)	19.98 (1.63)	1.13 (0.23)	1.15 (0.10)	7.26 (1.46)	23.07 (1.25)	0.31 (0.06)
<i>R. ser</i>	Oblik	23.31 (1.39)	19.97 (1.22)	1.10 (0.24)	1.17 (0.11)	6.91 (1.06)	22.39 (1.18)	0.31 (0.04)
<i>R. ser</i>	Mean	22.32 (1.95)	19.80 (1.58)	1.11 (0.23)	1.13 (0.13)	6.43 (1.41)	21.63 (1.95)	0.30 (0.06)

**Figure 2.** LM photographs of pollen grains: 1- *R. myconi*, 2- *R. nathaliae*, 3- *R. serbica*, 4- hybrid individuals' 3-colporate pollen, 5- hybrid individuals' 9-colporate pollen, 6- hybrid individuals' 12-colporate pollen; a- equatorial view, b- polar view. Scale bar = 10  $\mu\text{m}$  for all photographs.**Table 4.** Pollen morphology of hybrid individuals expressed as mean value  $\pm$  SD (3p: 3-colporate pollen grains, 9p: 9-colporate pollen, 12p: 12-colporate pollen). All values in  $\mu\text{m}$ , except for P/E and d/D.

Species	Locality	P	E	Exine	P/E	d	D	d/D
Hyb 3p	R. Kamen	20.93 (1.53)	16.91 (1.32)	1.12 (0.20)	1.24 (0.12)	5.39 (1.04)	20.35 (1.40)	0.27 (0.05)
Hyb 9p	R. Kamen	26.07 (2.91)	21.83 (2.35)	1.06 (0.21)	1.20 (0.10)			
Hyb 12p	R. Kamen	27.43 (2.99)	23.34 (2.32)	1.08 (0.16)	1.18 (0.11)			
Hyb 3p	Oblik	20.87 (1.05)	15.07 (1.35)	1.08 (0.18)	1.40 (0.14)	5.26 (0.85)	19.55 (1.38)	0.27 (0.04)
Hyb 9p	Oblik	25.57 (3.65)	19.87 (2.70)	0.96 (0.21)	1.29 (0.13)			
Hyb 12p	Oblik	26.33 (2.70)	21.09 (1.84)	0.96 (0.19)	1.26 (0.15)			

Nested-design ANOVA showed that most of the pollen characteristics were statistically significant ( $P < 0.01$ ) among populations as well as among species. However, dimensions of polar axis and equatorial diameter provide the basis for differentiating the 3 species (Table 5).

Pollen grains from all 3 species are tricolporate (Figures 2 and 3). The porus is not always clearly visible and the colpium surface is ornamented. In hybrid individuals, 3 types of pollen grains are found (on the basis of the number of apertures): 3-, 9- and 12-colporate. All 3 types were found in all investigated hybrid individuals. Three-colporate pollen grains are more frequent and they make up approximately 82% of pollen grains, while 9-colporate are 8% and 12-colporate are approximately 10%. Exine ornamentation is microreticulate to perforate in *R. myconi* and *R. nathaliae*, while it is microreticulate to almost bireticulate in *R. serbica* and hybrids (Table 6; Figure 3). Significant differences in lumen size between the apo- and mesocolpium regions were observed only in *R. myconi* ( $F = 20.46$ ,  $P = 0.000058$ ).

Pollen viability is high in all 3 species (Figure 4). The only exception is an *R. nathaliae* population on serpentine from the gorge of the river Pčinja. In hybrid individuals the viability of pollen is lower than in the parental species and around 11%–16% higher for 9- or 12-colporate than for normal 3-colporate grains.

### 3.3. Seed morphology and germination capacity

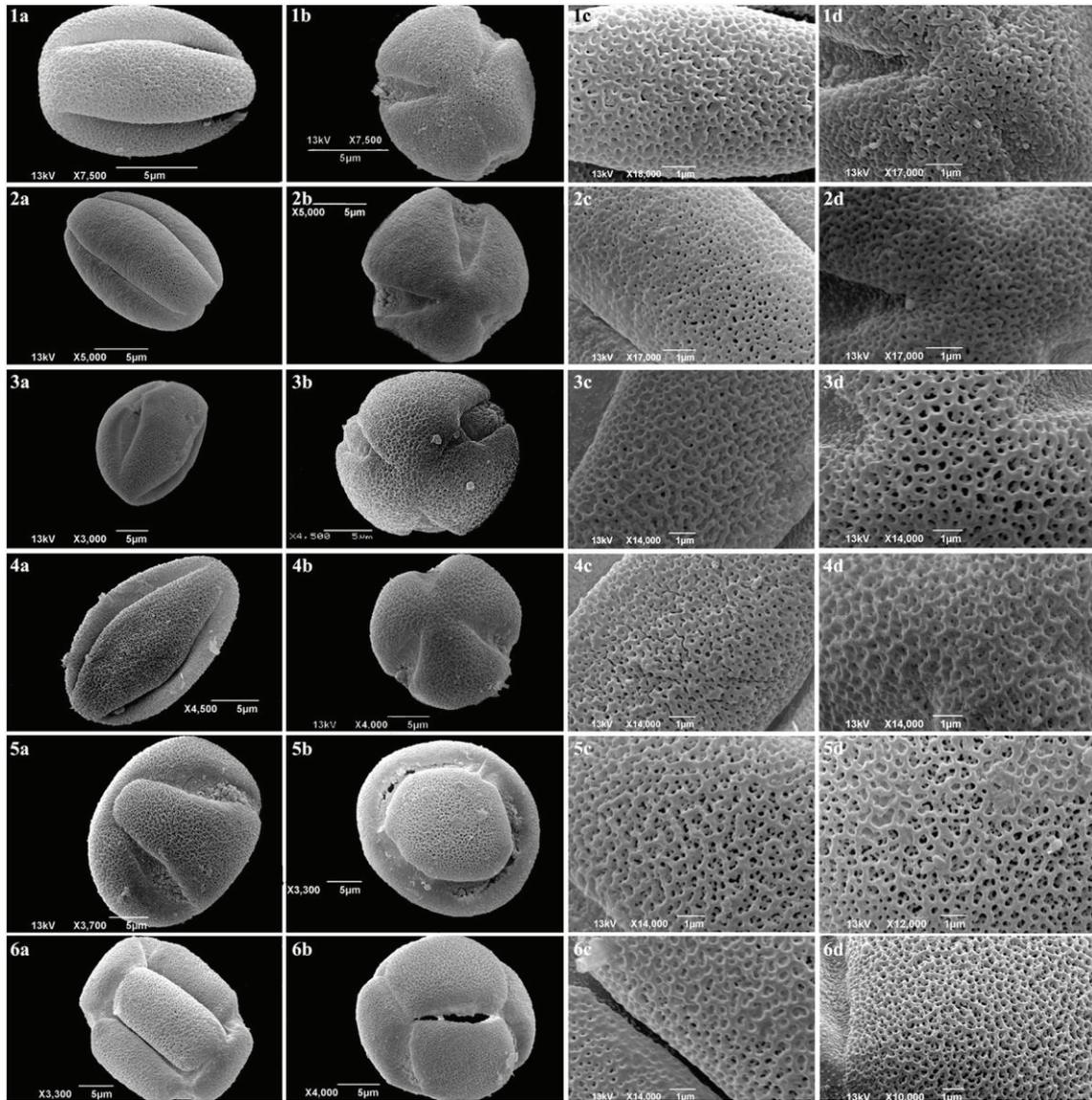
Seeds are numerous, small, less than 1 mm long and 0.5 mm wide, ellipsoidal, and dark brown (Table 7; Figure 5). The longest seeds, with the greatest length-to-width ratio, are those from *R. myconi*. The Balkan species have the same length-to-width ratio, but *R. serbica* has longer and wider seeds than *R. nathaliae*. The serpentine population of *R. nathaliae* has the smallest seeds. Seeds from hybrid individuals are very small, 2–2.5 times shorter and 3–3.5 times narrower than the seeds from parental species. There are significant differences between species, as well as between different populations of the same species, primarily in terms of seed length (Table 5).

Since the seeds are very light, we measured 5 batches of 100 seeds and then calculated the average mass of a single seed (Table 7). *Ramonda myconi* seeds were lightest and *R. serbica* the heaviest. Among *R. nathaliae* populations, individuals from sympatric populations had heavier seeds than those from localities where they grow alone. On the other hand, *R. serbica* individuals from sympatric populations had seeds that were slightly lighter than those collected from unmixed natural populations.

The seed coat surface is rough, reticulate, and ornamented (Figure 6). Lumens are elongated and visible in all specimens. They are largest in *R. myconi* and smallest in *R. nathaliae* (Table 8). The ratio of seed length to lumen

**Table 5.** F-values from one-way ANOVA (values in bold are  $P < 0.01$ ) and factor loadings for first 3 axes from PCA (marked loadings are  $>0.7$ ) for measured characteristics in investigated *Ramonda* populations.

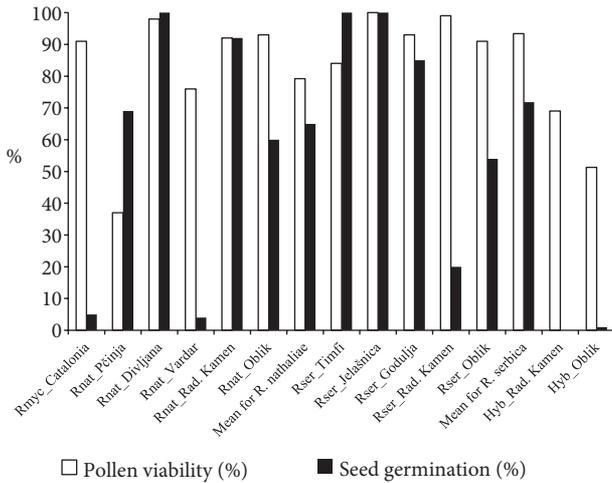
	F		PCA factor loadings		
	Species	Populations	PCA1	PCA2	PCA3
P axis	<b>231.11</b>	<b>10.44</b>	<b>-0.837</b>	-0.287	0.037
E axis	<b>154.51</b>	<b>12.60</b>	<b>-0.776</b>	0.067	-0.019
Exine thickness	<b>8.83</b>	0.94	0.004	0.227	-0.264
d	<b>33.52</b>	<b>5.28</b>	<b>-0.753</b>	0.371	0.376
D	<b>301.92</b>	<b>14.78</b>	<b>-0.791</b>	0.338	0.042
d/D	1.48	<b>3.84</b>	-0.371	0.212	0.498
P/E	1.63	<b>5.93</b>	-0.211	-0.575	0.086
Seed length	<b>9.42</b>	<b>6.59</b>	0.340	<b>0.786</b>	-0.007
Seed width	1.31	<b>7.20</b>	0.318	<b>0.897</b>	-0.012
L/W	<b>5.97</b>	<b>2.43</b>	-0.198	-0.627	0.013
Seed mass	<b>21.23</b>	<b>16.61</b>	-0.219	0.197	-0.553
2C (pg)	<b>4745.46</b>	<b>3.35</b>	-0.446	0.116	-0.692
Cx (pg)	<b>79.97</b>	<b>3.45</b>	-0.302	-0.074	-0.616
2n			<b>-0.817</b>	0.354	0.087



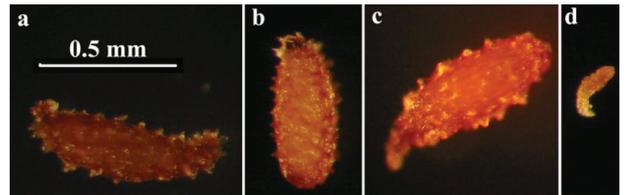
**Figure 3.** SEM microphotographs of pollen grains: 1- *R. myconi*, 2- *R. nathaliae*, 3- *R. serbica*, 4- hybrid individuals' 3-colporate pollen, 5- hybrid individuals' 9-colporate pollen, 6- hybrid individuals' 12-colporate pollen; a- equatorial view, b- polar view, c- details of exine ornamentation in mesocolpial region, d- details of exine ornamentation in apocolpial region.

**Table 6.** Exine ornamentation characteristics of 3 *Ramonda* species and hybrid individuals expressed as mean values (SD in parentheses). P: polar area, E: equatorial area.

Species	Lumen width ( $\mu\text{m}$ )		Murus width ( $\mu\text{m}$ )		No. of perforations per lumen		No. of lumens/ $25 \mu\text{m}^2$	
	P	E	P	E	P	E	P	E
<i>R. myconi</i>	0.14 (0.06)	0.24 (0.07)	0.18 (0.03)	0.15 (0.04)	1–4	1–3	149	221
<i>R. nathaliae</i>	0.14 (0.04)	0.12 (0.04)	0.13 (0.03)	0.14 (0.03)	1–6	1–3	330	>330
<i>R. serbica</i>	0.36 (0.11)	0.37 (0.12)	0.19 (0.03)	0.18 (0.05)	1–5	1–4	116	99
Hybrids 3p	0.29 (0.06)	0.24 (0.10)	0.18 (0.03)	0.19 (0.04)	1–3	1–3	114	170
Hybrids 9p	0.39 (0.11)	0.31 (0.09)	0.20 (0.04)	0.20 (0.06)	1–4	1–5	104	100
Hybrids 12p	0.37 (0.09)	0.34 (0.10)	0.20 (0.05)	0.14 (0.04)	1–4	1–4	136	160



**Figure 4.** Percentage of pollen viability and seed germination in studied populations of *Ramonda*.



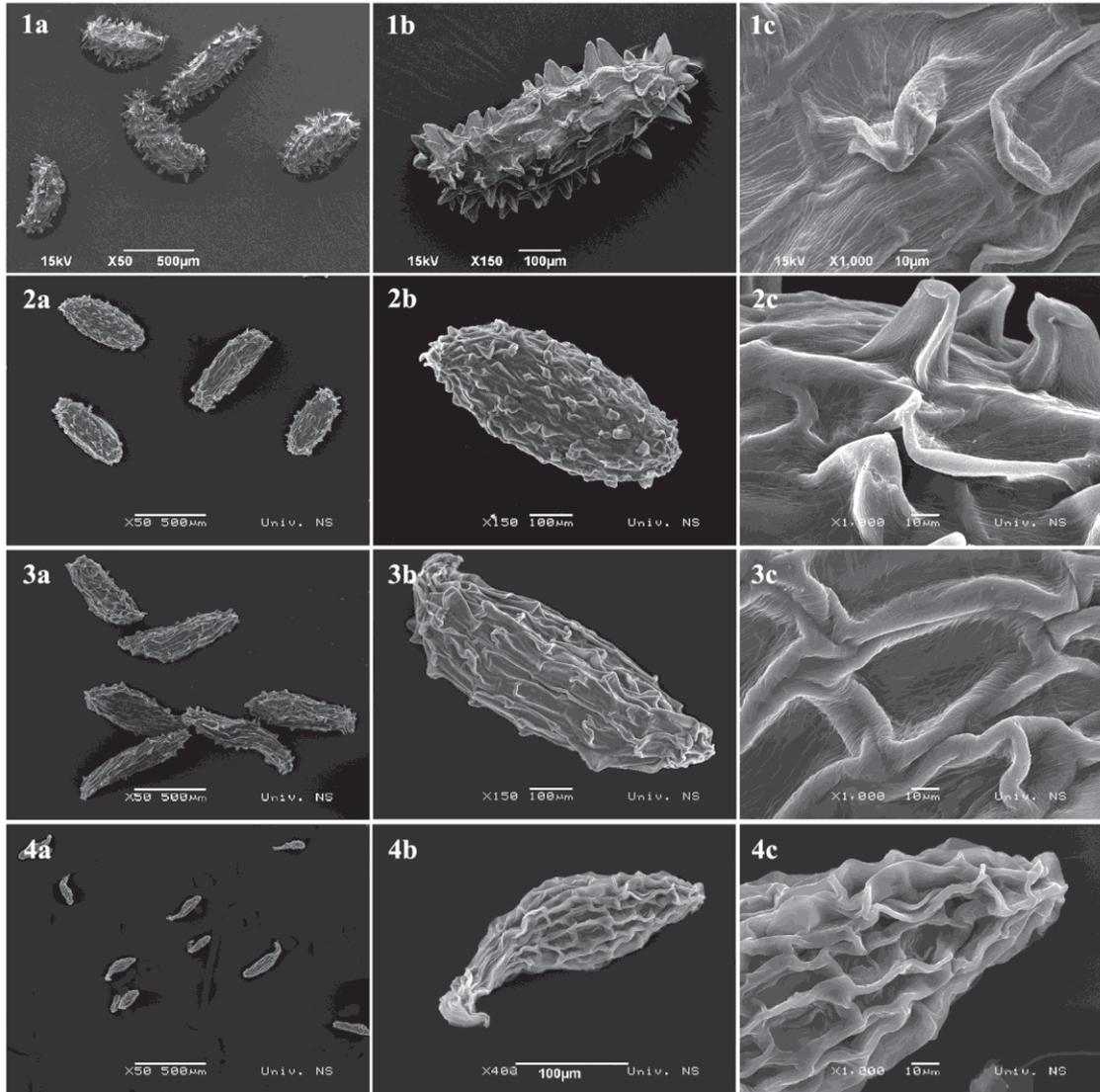
**Figure 5.** LM photographs of *Ramonda* seeds: a- *R. myconi*, b- *R. nathaliae*, c- *R. serbica*, d- hybrid. Scale bar 0.5 mm for all photographs.

**Table 7.** Seed morphology of 3 *Ramonda* species and hybrid individuals (mean value (±SD)).

Taxon	Population	Length (µm)	Width (µm)	Length/Width	Seed weight (µg)
<i>R. myc</i>	Sp: Enseu	814.47 (81.57)	217.77 (38.88)	3.89 (0.97)	17.4 (1.52)
<i>R. nat</i>	Mac: Pčinja	450.95 (102.58)	158.68 (28.54)	2.93 (0.87)	16.0 (2.00)
	Srb: Divljana	785.85 (68.68)	230.44 (20.14)	3.44 (0.46)	19.6 (0.89)
	Mac: Vardar	584.10 (70.95)	197.28 (28.26)	3.00 (0.45)	9.8 (2.49)
	Srb: R. Kamen	682.75 (71.44)	278.84 (35.52)	2.48 (0.39)	21.6 (3.21)
	Srb: Oblik	692.51 (94.44)	275.20 (53.41)	2.56 (0.38)	23.33 (1.15)
	Mean value	639.23 (140.52)	228.09 (57.7)	2.88 (0.64)	18.07 (5.37)
	<i>R. ser</i>	Gr: Timfi	746.61 (67.95)	275.32 (25.41)	2.73 (0.33)
Srb: Jelašnica		649.13 (88.26)	250.59 (32.27)	2.64 (0.55)	20.8 (1.09)
Srb: Godulja		752.23 (72.43)	265.90 (25.30)	2.86 (0.40)	25.6 (1.14)
Srb: R. Kamen		734.90 (104.37)	242.32 (45.12)	3.15 (0.81)	20.8 (1.30)
Srb: Oblik		688.93 (105.17)	240.06 (56.16)	3.05 (0.98)	17.6 (5.90)
Mean value		714.36 (96.74)	254.84 (40.83)	2.88 (0.68)	22.48 (4.04)
<i>Hyb</i>	Srb: R. Kamen	284.47 (58.12)	74.75 (13.46)	3.88 (0.85)	
	Srb: Oblik	297.87 (51.03)	72.96 (14.27)	4.18 (0.84)	
	Mean value	291.17 (55.07)	73.86 (13.89)	4.03 (0.85)	

length is similar in *R. myconi* and *R. serbica* (7.26 and 6.90). In *R. nathaliae* (8.63) and especially in hybrid individuals (9.45) it is much larger, indicating the smaller lumens. The seed surface is similar in the 3 species. There are slight differences in details, such as the outline of cells that are

tetra- to hexagonal in *R. myconi* and *R. nathaliae* and tetra- to pentagonal, rarely trigonal in *R. serbica*. Additional structures of auriculate type are present irregularly on the seed surface of all 3 species and are especially distinct in *R. myconi* (Table 8). They are usually absent in hybrid



**Figure 6.** SEM microphotographs of *Ramonda* seeds: 1- *R. myconi*, 2- *R. nathaliae*, 3- *R. serbica*, 4- hybrid individuals; scale bars: a- 500 µm, b- 100 µm, c- 10 µm.

**Table 8.** Seed ornamentation characteristics in 3 *Ramonda* species and hybrid individuals (mean ± SD, in µm).

Species	Lumen length	Lumen width	Murus width	Ornamentation height
<i>R. myconi</i>	112.16 (12.09)	40.11 (5.36)	6.97 (1.20)	64.27 (12.50)
<i>R. nathaliae</i>	74.11 (16.81)	24.31 (6.24)	5.63 (1.23)	26.54 (3.22)
<i>R. serbica</i>	103.50 (24.43)	33.01 (9.69)	6.16 (1.45)	37.58 (3.34)
Hybrids	30.91 (11.10)	11.63 (4.73)	2.79 (0.85)	

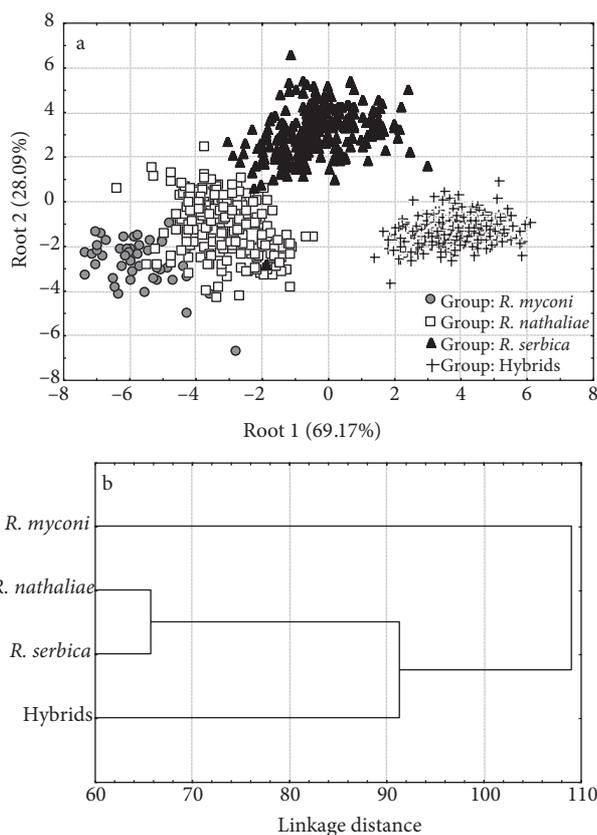
individuals. Their seeds look more wrinkled with much more expressed lumen depth, so there are only indications of the presence of auriculates in some lumens.

Seed germination rates are variable but quite high in the Balkan *Ramonda* species (Figure 4). In *R. nathaliae*, for example, populations from Oblik and the river Pčinja

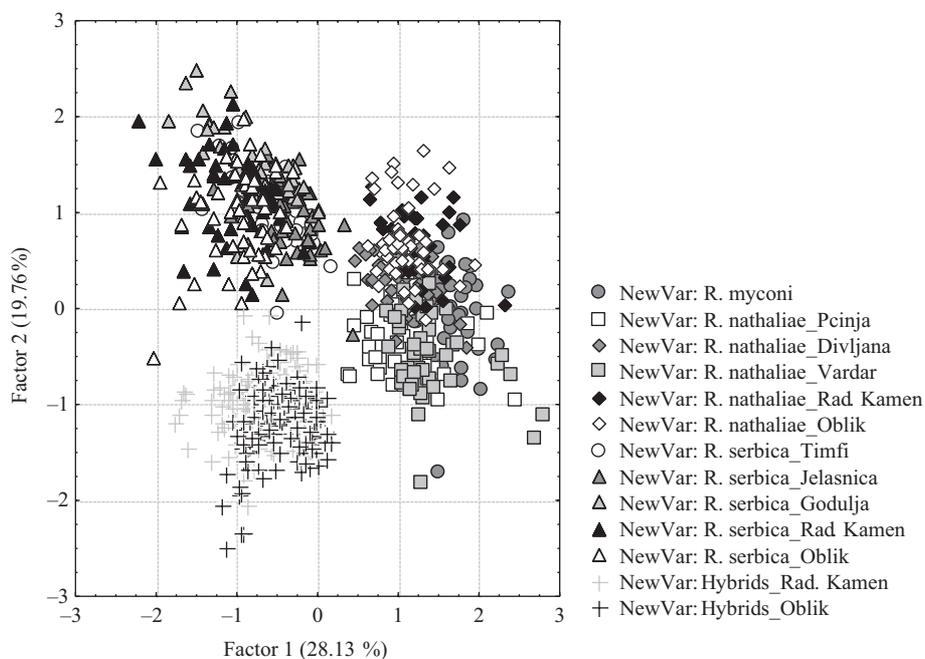
gorge had somewhat lower germination rates (60% and 69%, respectively), while seeds from the gorge of the river Vardar had a germination success of only 4%. Among populations of *R. serbica*, lower germination rates are evident in individuals in both sympatric populations. *Ramonda myconi* seeds germinated weakly (only 5%), and those from hybrid individuals even less (1%).

PCA, with all characters selected, showed that the first axis is responsible for 28.13%, the second for 19.76%, and the third for 11.73% of the variation. Factors that contributed most to variations along the first axis are the dimensions of the polar and equatorial axes, distances between the apices of 2 ectocolpi, the equatorial axis in polar view, and chromosome number (Table 5). Characters contributing to differentiation along the second axis are seed length and width. A scatterplot graph (Figure 7) shows gathering of *R. myconi* and *R. nathaliae* on the left side of the first axis and *R. serbica* and hybrid individuals on the right side. The second axis, where the influence of seed characters is predominant, highlights the differentiation of hybrid individuals from *R. serbica* and the slight grouping of *R. nathaliae* populations from Macedonia.

In CDA, the data were treated regardless of populations but only related to species. Four groups stand out (Figure 8a). The first group is made up of *R. myconi*, settled close to *R. nathaliae*, a diploid from the Balkans that forms the second group. *Ramonda serbica* constitutes a third group, slightly overlapping with *R. nathaliae*, while hybrid individuals are clearly differentiated and form the fourth



**Figure 8.** Results of CDA plotted along the first 2 discriminant axes (a) and cluster analysis (UPGA) based on Mahalanobis distances (b) for 3 *Ramonda* species and hybrid individuals.



**Figure 7.** Results of PCA plotted along the first 2 discriminant axes for all characteristics investigated in *Ramonda* populations.

group. Cluster analysis based on Mahalanobis distances made the separation of the 3 species more evident (Figure 8b). The Balkan species are linked more closely to each other and to hybrid individuals, while the Pyrenean *R. myconi* forms a separate group.

Several characteristics are significantly correlated statistically. However, only equatorial axis in the polar view (D) was strongly correlated to chromosome number ( $r = 0.84$ ,  $P = 0.00$ ) and 2C-value ( $r = 0.78$ ,  $P = 0.00$ ), while other pollen (P, E) and seed traits (primarily seed mass) showed statistically significant, but only moderate, correlation to these parameters (Table 9).

#### 4. Discussion

This first detailed comparative morphological study of pollen and seeds in the genus *Ramonda* provides additional information for their distinction and phylogeny, but also for their ecology. The pollen of *Ramonda* species' with its small, 3-colporate grains, spheroidal form, and circular outline fits completely into the general description of pollen of the family Gesneriaceae (Weber, 2004). The pollen grains in 2 diploid species, *R. myconi* and *R. nathaliae*, are very similar regarding size and shape, the mean values for their polar axes being 14.24 and 16.75  $\mu\text{m}$ . Only in *R. serbica* does the pollen size tend to be larger, with a mean value of 22.32  $\mu\text{m}$  and maximal value of 28  $\mu\text{m}$ , which could be related to the larger genome size. Exine ornamentation clearly separates *R. nathaliae* from *R. serbica*.

In general, the genus *Ramonda* is characterized by dry capsular fruits and dehiscence is performed mostly by longitudinal slits. Small seeds are produced in large numbers and are not specialized for any specific mode of dispersal. They fall freely on surrounding rocks and mosses although, since the seeds are small and light, the wind can also play a role in their dispersal. There is a high

level of overlap in the ranges of seed size of the 3 species studied. Variation is especially great in *R. nathaliae*, probably due to the greater variety of habitats of this species. On the other hand, seed mass is similar in the 2 diploids, *R. myconi* and *R. nathaliae*, and smaller than in hexaploid *R. serbica*, showing a moderate correlation of this parameter to genome size and chromosome number, but not statistically significant after Bonferroni correction of P-value. In all 3 species, auriculate ornamentations were observed on the seed surface. These features are especially striking in *R. myconi* and, since they are of different sizes in the 3 species, can be used as a useful parameter for their differentiation. It is interesting that it has been suggested that European Gesneriaceae genera produce seeds without testa cell ornamentations (Möller et al., 2009), as is the case with *Haberlea* Friv. and *Jankaia* Boiss. (Beaufort-Murphy, 1983). However, we have shown that representatives of the genus *Ramonda* possess auriculate ornamentation on the seed surface, and similar structures can be found in some representatives of African (*Streptocarpus* Lindl.) and Asiatic genera (*Trisepalum* C.B. Clarke, *Cyrtandra* J.R. Forst. & G. Forst.) (Beaufort-Murphy, 1983). This type of ornamentation could help seeds attach to the surface of rocks or mosses. Heavier surface sculpturing could also contribute to the increased thermodynamic exchange in plants growing under water/temperature stress (Barthlott, 1981), as is the case with representatives of the genus *Ramonda*.

Both pollen viability and seed germination capacity are generally high in the examined species, contributing to the high reproductive potential of their populations. The main exception is the very low seed germination of *R. myconi*. However, this could be explained by inadequate storage of seeds of this species before the beginning of the experiment, which could have influenced the final germination success (Mancuso et al., 2012).

##### 4.1. Sympatric populations and hybrids

There are several factors that contribute to the existence of natural hybridization between *R. nathaliae* and *R. serbica*. Although their populations are clearly separated throughout their ranges, there are 2 localities where they live together and where hybrids are found. They have similar flowers that blossom at the same time and are pollinated by the same species of bumble bee, such as *Bombus haematurus* Kriechbaumer 1870 and *Bombylus* sp., but possibly also by some other insects. An experiment in which flower buds were covered by tulle fabric bags showed that *Ramonda* plants are most probably self-incompatible, since none of them produced seeds under this condition. The existence of hybrid individuals between the 2 Balkan species suggests that, although they are clearly separated, there are still no completely effective barriers for their intercrossing, as is the case in some other representatives of Gesneriaceae

**Table 9.** Correlation coefficients of different characters measured in pollen and seeds and parameters of genome size in *Ramonda*. Correlations marked by \* are significant at  $P < 0.01$  and those marked by \*\* at  $P < 0.000128$  (P-value after Bonferroni correction).

	2C (pg)	Cx (pg)	2n
P	0.34**	0.28**	0.59**
E	0.31**	0.26**	0.61**
D	0.78**	0.61**	0.84**
Seed length	-0.08	-0.28**	0.02
Seed width	-0.10	-0.32**	0.07
Seed mass	0.45*	0.18	0.47*

(Kvist and Skog, 1992; Fuchs et al., 2011). Their large 9- and 12-colporate pollen grains are most probably polyads formed during irregular microsporogenesis. The capacity of the hybrids' seeds for germination is very low: only about 1%. However, since many hybrid individuals are found in sympatric populations, it is likely that while the first generation of hybrids is established from the seeds, vegetative reproduction becomes dominant in their further reproduction.

#### 4.2. Correlations between pollen and seed characters and genome size

Strong correlations were observed between pollen size and chromosome number and between pollen size and 2C DNA content. However, seed size parameters did not show significant correlations with ploidy level. Only seed mass was moderately correlated to chromosome number and 2C DNA content. Thus, while pollen size may be used cautiously for determining the ploidy level in *Ramonda*, seed size cannot be used for this purpose, because of the partial overlapping of the size ranges. This is not unusual, since the relationship between genome size and, for example, seed mass is complex and not linear (Beaulieu et al., 2007). Since seed size and weight can be closely related to habitat ecology (Brochmann, 1992), it could be that, in this case, environmental influence on seed characteristics prevailed over ploidy level.

#### 4.3. Influence of environment on pollen and seed characteristics

Although the ecological requirements of all 3 *Ramonda* species are very similar, there are some differences in their habitats. Specifically, there are certain populations of *R. nathaliae* that can be found on substrate types other than limestone, such as serpentine, granite, or schist (Košanin, 1921; Stevanović and Stevanović, 1985). These habitats are usually characterized by harsher environmental conditions, especially by more intense sunlight and longer lasting, more severe drought. In this sense, it is interesting to consider the *R. nathaliae* population from the gorge of the river Pčinja that grows on serpentine substrate. This locality is exposed to sunlight and covered with sparse vegetation tolerant to extreme serpentine soil conditions, such as low Ca-to-Mg ratio, lack of essential nutrients, and high concentrations of certain heavy metals (Brooks, 1987; Brady et al., 2005; Altınözlü et al., 2012). Despite these general conditions, there are a number of relatively protected northward-facing micro sites, like stony gaps and crevices, that can provide sufficient relief from the otherwise inhospitable climatic and soil conditions. In these circumstances, *R. nathaliae* plants are smaller and have the smallest pollen grains, the

thinnest exine, the lowest pollen viability, and the smallest seeds. They are also characterized by the smallest genome size. However, when such plants were transferred from their natural habitat to the Botanical Garden in Belgrade, they produced highly viable pollen grains (95%). This suggests that at least some of the inhospitable environmental conditions of the serpentine habitat have negative effects on the pollen viability of this facultative serpentinophyte. Several reports have shown that ploidy can increase tolerance to environmental stress through a stronger antioxidant defense system and increased heat tolerance (Zhang et al., 2010). It would be interesting to explore whether this is the case with hexaploid *R. serbica* compared to diploid *R. nathaliae*.

Our results show that relationships between the 3 clearly separated species are very complex regarding genome size and characteristics of pollen and seeds. The similar quantitative characteristics of their pollen grains, influenced by genome size, are linking the 2 diploids closer together. Exine ornamentation shows clear differences between *R. nathaliae* and *R. serbica*, while *R. myconi* exhibits similarities to both Balkan species. Both quantitative and qualitative characteristics of seeds link *R. nathaliae* and *R. serbica* closer together, but at the same time show that *R. myconi* has seeds that are more similar to the Balkan hexaploid than to the diploid species. Different hypotheses about the origin and relationships of these species have been proposed (Košanin, 1921; Tatić and Stefanović, 1976; Stevanović and Stevanović, 1985; Siljak-Yakovlev et al., 2008), but only further and primarily molecular analyses combined with the already known morphological, phytocoenological, and ecological characteristics of the 3 species will provide a more precise answer.

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