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Genome size variation and polyploidy in the resurrection plant genus *Ramonda*: Cytogeography of living fossils

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Abstract

The genus *Ramonda* includes three preglacial paleoendemic species surviving as the rare resurrection angiosperms of the Northern hemisphere in refugia habitats in the Balkan (*Ramonda nathaliae* and *Ramonda serbica*) and Iberian Peninsulas (*Ramonda myconi*). This study focuses on: assessing genome size and base composition, determining chromosome number and ploidy level in several populations, evaluating inter- and intra-specific variations in DNA content and chromosome number as well as looking for the possible hybridization in the sympatric zones of Balkan species. *R. nathaliae* and *R. myconi* are diploid species ($2n = 2x = 48$) while *R. serbica* is hexaploid ($2n = 6x = 144$). The mean 2C DNA values ranged from 2.30 pg for *R. nathaliae* to 2.59 pg for *R. myconi* compared to 7.91 pg for *R. serbica*. The base composition for *R. nathaliae* was 42.1% GC, for *R. myconi* 39.9% and for *R. serbica* 41.2%. In one population of *R. serbica* the DNA content ranged from 2C = 7.65 to 11.82 pg, revealing different ploidy levels among its individuals. In sympatric populations genome size was intermediary (∼5 pg) between the diploid and hexaploid classes which indicates the hybridization ability between *R. serbica* and *R. nathaliae*. It appears that polyploidization is the major evolutionary mechanism in the genus *Ramonda*.

Keywords: Paleopolyploids; Resurrection plants; *Ramonda*; Sympatric populations; Tertiary relicts; Cytometry

1. Introduction

Three closely related poikilohydric or resurrection plants, *Ramonda serbica* Panč., *Ramonda nathaliae* Panč. et Petrov. and *Ramonda myconi* (L.) Reich., survive as preglacial relicts and paleoendemic species on specific refugia habitats in the Balkan and Iberian Peninsulas. They belong to the pantropic family of Gesneriaceae represented in Europe by three genera and five species (three *Ramonda* species, *Haberlea rhodopensis* and *Jankaea heldreichii*), all as resurrection plants. Four of these species occur in SE Europe, and the only representative in SW Europe is *R. myconi*. As survivors of successive glaciations during the European Ice Age, the *Ramonda* species manifest a fragmented distribution in the Balkans, as well as in the Iberian Peninsula. Their current distribution is restricted to the northern rocky slopes of gorges and canyons, mainly on foothills, reaching sometimes the alpine belts (Kosanin, 1921; Meyer, 1970). They all prefer limestone rocks, while *R. nathaliae* also settles on ophiolitic bedrock (Kosanin, 1921; Stevanovic and Stevanovic, 1985).

Ranges of the *Ramonda* species in the Balkans overlap in SE Serbia, constituting a sympatric zone with mixed or spatially very close populations (Stevanovic et al., 1986b). Hence, distribution of *R. serbica* extends from NW Bulgaria, SE and SW Serbia, NE and SE Montenegro to Albania, W FYR Macedonia and NW Greece, while range of *R. nathaliae* is limited to N and C FYR Macedonia and N Greece, with a disjunct part of the

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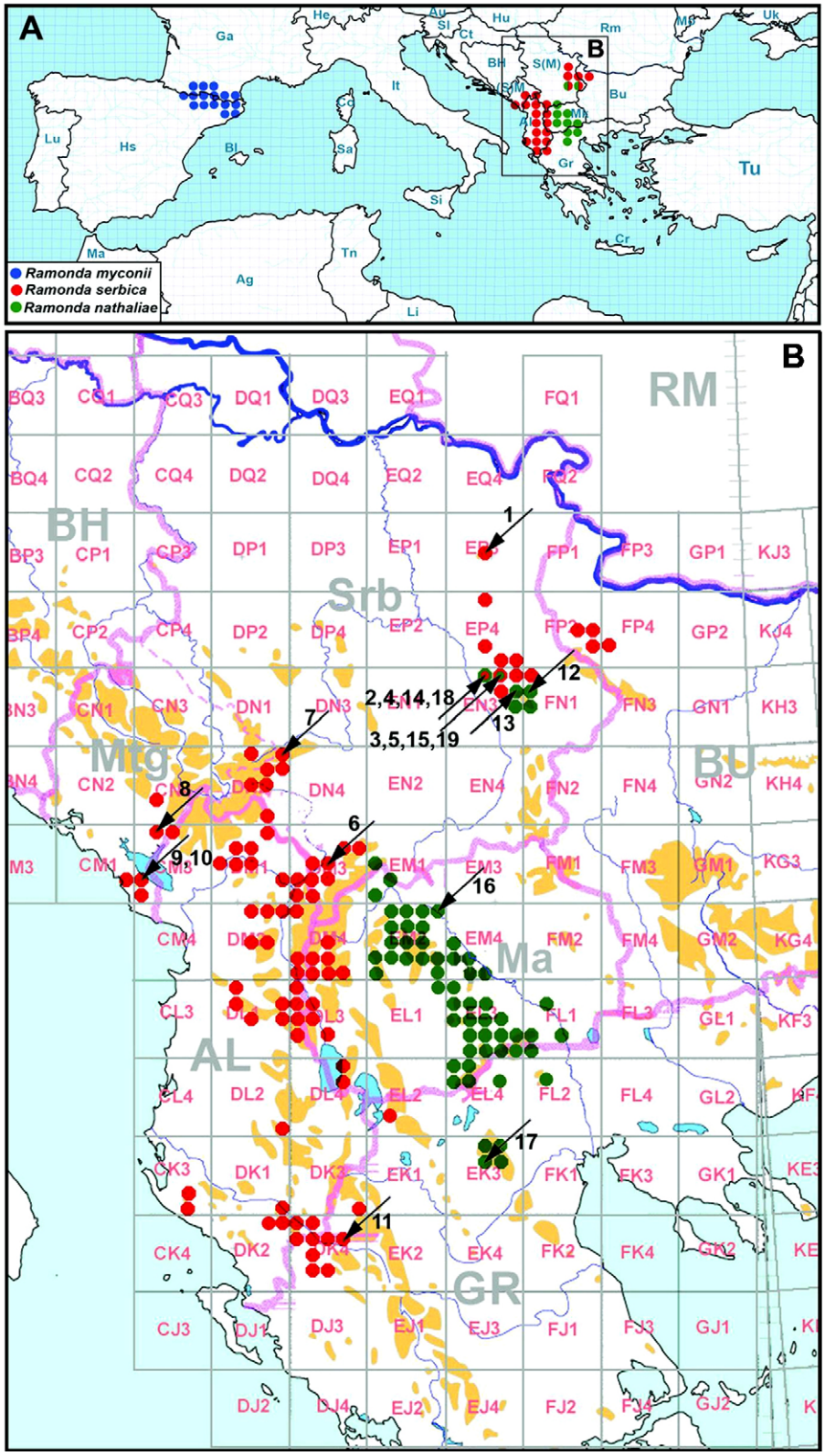

Fig. 1. Distribution of the genus *Ramonda* presented at UTM grids 50 km × 50 km (A). Abbreviations of European countries (territories) are given according to Atlas Florae Europaeae 13 (Kurtto et al., 2004). General distribution of *Ramonda serbica* (red dots) and *R. nathaliae* (green dots) in the Balkans presented at UTM grids 10 km × 10 km (B). Arrows indicate localities of investigated populations of both *Ramonda* species. Numbers of localities corresponded to those listed in Table 1.

range in SE Serbia (Kosanin, 1921; Meyer, 1970; Stevanovic et al., 1986a,b, 1991) (Fig. 1).

The range of *R. myconi* is restricted to Iberian Peninsula (Prepyrenean and Pyrenean mountains and Catalan Prelittoral range) where it thrives in limestone gorges and humid mountain valleys (Meyer, 1970; Bolos and Vigo, 1995).

The Balkan *Ramonda* species were discovered at the beginning of 19th century by Serbian botanists Pancic and Petrovic. Their taxonomy, phytogeography, ecology, embryology and ecophysiology have since been studied (Pancic, 1874; Petrovic, 1885; Kosanin, 1921, 1939; Glisic, 1924; Stefanoff and Georgiev, 1937; Micevski, 1956; Quezel, 1968; Meyer, 1970; Jankovic and Stevanovic, 1981; Stevanovic and Stevanovic, 1985; Stevanovic et al., 1986a,b, 1987, 1991; Stevanovic, 1986, 1989; Stevanovic et al., 1992; Stevanovic and Glisic, 1997). However, cytogenetic data on the genus are still very scarce.

Recent literature has largely addressed mechanisms of genome size evolution (Bennetzen, 2002; Jakob et al., 2004; Bennetzen et al., 2005; Gregory, 2005). The value of Cx (monoploid C-value according to Greilhuber et al., 2005) is particularly interesting when studying species complexes in which polyploidy occurs. The desiccation-tolerant *Ramonda* can be a good model for such investigations because (i) it comprises many isolated populations across a fragmented distribution, (ii) different ploidy levels exist and (iii) sympatric populations of two Balkan species are available. Given the paucity of information on genome size in *Ramonda* populations – only one C-value concerning *R. myconi* (Zonneveld et al., 2005) – we have measured the DNA content of 19 populations belonging to the three species, as well as two populations where *R. nathaliae* and *R. serbica* grow in sympatry.

The chromosome numbers of these species were determined earlier by Glisic (1924), Ratter (1963) and Contandriopoulos (1966) but the reported data are not in accordance. Therefore, the chromosome number has been verified and completed in this study. This parameter gives an early indicator of polyploidy series, hybridization and finally speciation in a complex of related species.

The investigation presented here involved examination of: (1) the genome size and base composition in the genus *Ramonda*, (2) chromosome number and ploidy level in several populations of three *Ramonda* species, (3) inter- and intra-specific variations in DNA content and chromosome number, (4) possible hybrids in the sympatric zone and (5) demographic and cytogeographic data concerning several populations of *R. serbica* and *R. nathaliae* species growing in gorges and mountains in the Balkans (especially in Serbia and in Montenegro), the geographical region which is considered as a hot spot of plant diversity in Europe (Stevanovic et al., 2004).

2. Material and methods

2.1. Plant material

Sample sites included 2 populations of *R. myconi* (syn.: *R. pyrenaica* Pers.) from Spain, 6 populations of *R. nathaliae* (4 from Serbia, 1 from Greece and 1 from FYR Macedonia), 11 populations of *R. serbica* (7 from Serbia, 3 from Montenegro and 1 from Greece) and 2 populations where *R. serbica* and *R. nathaliae* grow in sympatry (Table 1 and Figs. 1A and B and 2A–C).

Material for DNA content analysis was collected from different parts of the range of the species. Detailed descriptions of these habitats including coordinates of localities, type of substrata, chasmophytic community, are given in Table 1. Vouchers of the studied populations are deposited in the Herbarium of Institute of Biology and Botanical Garden (BEOU), Department of Plant Ecology and Phytogeography, Faculty of Biology, University of Belgrade.

General distribution of the *Ramonda* species was mapped at UTM grid 50 km × 50 km, whereas the detailed distribution of these species was given at UTM grid 10 km × 10 km (Fig. 1A and B).

2.2. Chromosome preparation

For mitotic chromosome analysis, root-tip meristems obtained from plants in pots were pre-treated with 0.002 M 8-hydroxyquinoleine for 4.5 h at 16 °C. Fixation was performed in cold 3/1 (v/v) ethanol/acetic acid at least 24–48 h. After hydrolysis in 1N HCl 14 min at 60 °C and staining in Schiff reagent following standard Feulgen and Rossenbeck (1924) method, the squash was realized in a drop of acetic carmine. After freezing at −80 °C during 24 h, cover slips were removed, preparations were dried at least 24 h and then mounted in Euparal.

Chromosome plates were observed under the Zeiss Axiophot microscope and analysed using a CCD camera (Princeton Instruments, Evry, France) and image analyser (Metavue). Chromosome number was determined from at least 5 individuals per population and from at least 25 cells per root tip.

2.3. Estimation of pollen viability

The pollen grains were stained in Alexander reagent (Alexander, 1969) which stains the normal pollen in red and the aborted pollen in green.

2.4. Estimation of nuclear DNA content and base composition by flow cytometry

The total DNA amount was assessed by flow cytometry according to Marie and Brown (1993). *Pisum sativum* cv. Long Express (Truffaut SA) (2C = 8.37 pg; 40.5% GC) and *Lycopersicon esculentum* cv. Roma (2C = 1.99 pg, 40.0% GC, Marie and Brown, 1993) were used as internal standards. Leaves of studied species and internal standard were chopped using a razor blade in a Petri dish with 600 μL of Galbraith et al. (1983) nucleus-isolation buffer, supplemented with 10 mM sodium metabisulfite and 1% polyvinylpyrrolidone 10,000. The suspension was passed through a 48 μm mesh nylon filter. The nuclei were stained with 50 μg/mL propidium iodide, a specific DNA fluorochrome intercalating dye, used after RNAse treatment (2.5 U/mL) at 4 °C. Base composition was assessed using AT-specific fluorochrome bisbenzimidine Hoechst 33342 (5 μg/mL;

Table 1
Geographic origin and description of the *Ramonda* populations

Population code	Species	Localities of populations	Altitude (m)	Latitude; longitude	Geological substrate	Ecological conditions of habitat	Voucher number	Collector
1	*R. serbica*	NE Serbia: Canyon of r. Lazareva Reka near village of Zlot	310	N44°01′38.1″; E21°57′16.2″	Limestone	North faced rocks. Community *Ceterato-Ramondaetum serbicae*. Surrounding forest vegetation—*Carpinus orientalis*	370/90 BEOU	D. Lakusic, G. Tomovic and M. Tomasevic
2	*R. serbica*	E Serbia: Gorge of river Jelasnica in vicinity of Niš	290	N43°16′56.2″; E22°03′47.1″	Limestone	North faced rocks. Community *Ceterachi-Ramondaetum serbicae*. Surrounding forest vegetation—*Carpinus orientalis*	10944 BEOU	V. Stevanovic, M. Niketic, G. Tomovic and M. Tomasevic
3	*R. serbica*	E Serbia: Gorge of r. Nišava, 20 km E from Niš	308.5	N43°18′38.1″; E22°10′53.3″	Limestone	North faced rocks. Community *Cetereto-Ramondaetum serbicae*. Surrounding forest vegetation—*Carpinus orientalis*	20639 BEOU	M. Tomasevic, B. Zlatkovic and V. Stevanovic
4	*R. serbica*	SE Serbia: Radovanski Kamen above village Čukljenik	574.5	N43°17′5.1″; E22°4′16.6″	Limestone	North faced rocks. Community *Cetereto-Ramondaetum serbicae*. Surrounding forest vegetation—*Carpinus orientalis*	20640 BEOU	M. Tomasevic, B. Zlatkovic and V. Stevanovic
5	*R. serbica*	SE Serbia: Gorge of r. Nišava in the vicinity of village Ostrovica—Oblik	279.8	N43°19′52″; E22°8′43.3″	Limestone	North faced rocks. Community *Cetereto-Ramondaetum serbicae*. Mixed deciduous forests	20634 BEOU	M. Tomasevic, B. Zlatkovic and V. Stevanovic
6	*R. serbica*	SW Serbia-Kosovo and Metochia province: Gorge of r. Prizenska Bistrica in the vicinity of village Sredska	ca. 550	N42°15′08.7″; E20°86′67″	Limestone	North faced rocks. Community *Musco-Ramondaetum serbicae*	2910 BEOU	S. Duraki
7	*R. serbica*	SW Serbia: Gorge of r. Crna Reka in vicinity of monastery Sv Peter ca. 8 km southwards from village Ribarici	1100	N42°56′46.3″; E20°28′09.7″	Limestone	North faced rocks inside of beech forests. Community *Musco-Polypodio-Ramondaetum serbicae*	20623 BEOU	V. Stevanovic and D. Lakusic
8	*R. serbica*	SE Montenegro: Canyon of river Cijevna in vicinity of village Šumice	350	N42°25′22.3″; E19°27′48.4″	Limestone	North faced rocks. Community *Geranio dalmatici-Ramondaetum serbicae*	776/90 BEOU	V. Stevanovic, B. Stevanovic and Z. Bulic
9	*R. serbica*	SE Montenegro: Mt. Rumija, N slopes toward Skadar lake, gorge of stream Kroni e Murici above village Murići	420	N42°08′32.5″; E19°13′09.9″	Limestone	NW faced rocks. Community *Cheilantho fragrantis-Ramondaetum serbicae*	19673 BEOU	M. Niketic, G. Tomovic, M. Tomasevic and P. Lazarevic
10	*R. serbica*	SE Montenegro: Mt. Rumija, N slopes toward Skadar lake, gorge of stream Kroni e Besit above village Murići	250	N42°09′19.7″; E19°12′48.4″	Limestone	NW faced rocks. Community *Cheilantho fragrantis-Ramondaetum serbicae*	9880 BEOU	M. Niketic, G. Tomovic, M. Tomasevic and P. Lazarevic
11	*R. serbica*	NW Greece: North Pindhos region: Canyon of r. Voidomatis near village Papango—Vikos	ca. 650	N40°00′00″; E20°54′39.6″	Limestone	North faced rocks. Community *Ramonda serbica-Silene scwarzenbergiana*	20629 BEOU	M. Niketic
12	*R. nathaliae*	E Serbia: slopes of Suva planina near village Divjane in vicinity of Bela Palanka	420	N43°10′18.6″; E22°18′09.8″	Limestone	North faced rocks surrounded by stands of *Carpinus orientalis*. Type of community *Musco-Ramondaetum nathaliae*	20638 BEOU	V. Stevanovic, M. Niketic, G. Tomovic, M. Tomasevic and B. Zlatkovic

13	*R. nathaliae*	E Serbia: Mt. Suva Planina, ca. 2 km westward from main mountain top Trem	1750	N43°11′06.8″; E22°10′00.7″	Limestone	North faced rocks. Community *Erysimo-Ramondaetum nathaliae* (*Erysimum comatum-Ramonda nathaliae*)	20019 BEOU	D. Lakusic
14	*R. nathaliae*	SE Serbia: Radovanski Kamen above village Čukljenik	576.7	N43°17′1.2″; E22°4′23.5″	Limestone	North faced limestone rocks. Community *Cetereto-Ramondaetum nathaliae*	20641 BEOU	M. Tomasevic, B. Zlatkovic and V. Stevanovic
15	*R. nathaliae*	SE Serbia: Gorge of r. Nišava in the vicinity of village Ostrovica—Oblik	418.2	N43°19′47.4″; E22°8′44.9″	Limestone	North faced rocks. Community *Cetereto-Ramondaetum serbicae ramondaetosum nathaliae*	20637 BEOU	M. Tomasevic, B. Zlatkovic and V. Stevanovic
16	*R. nathaliae*	N Macedonia: Gorge of river Pcinja near village Katlanovo—Taorska gorge	405	N41°52′30.5″; E21°42′54.8″	Serpentine	North faced rocks in mixed deciduous submediterranean woodland	20962 BEOU	G. Tomovic
17	*R. nathaliae*	NC Greece: Mt. Vermion gorge in vicinity of village Sella westward from Naoussa	ca. 1000	N40°35′05.4″; E22°01′28.5″	Limestone	North faced rocks. Community *Ramonda serbica-Campanula formanekiana*	20624 BEOU	M. Niketic
18	Sympatric populations of *R. serbica* and *R. nathaliae*	SE Serbia: Radovanski Kamen above village Čukljenik	574.5	N43°17′5.1″; E22°4′16.6″	Limestone	North faced rocks. Community *Cetereto-Ramondaetum serbicae ramondaetosum nathaliae*	20640 BEOU	M. Tomasevic, B. Zlatkovic and V. Stevanovic
19	Sympatric populations of *R. serbica* and *R. nathaliae*	SE Serbia: Gorge of r. Nišava in the vicinity of village Ostrovica—Oblik	418.2	N43°19′47.4″; E22°8′44.9″	Limestone	North faced rocks. Community *Cetereto-Ramondaetum serbicae ramondaetosum nathaliae*	20636 BEOU	M. Tomasevic, B. Zlatkovic and V. Stevanovic
20	*R. myconi*	Spain, Catalonia, Girona: Camprodon, footpath to St. Antoni, E Pyrenees	1000	N42°19′00.1″; E2°22′00.1″	Limestone	North faced rocks in the forest *Buxus sempervirens-Pinus sylvestris*	177a Personal herbarium	T. Garnatje and D. Roca
21	*R. myconi*	Spain, Girona: Camprodon, St. Antoni mountain. Rocks near the path E Pyrenees	1200	N42°19′55″; E2°22′19.7″	Limestone	North faced rocks in the forest *Buxus sempervirens-Pinus sylvestris*	177b Personal herbarium	T. Garnatje and D. Roca

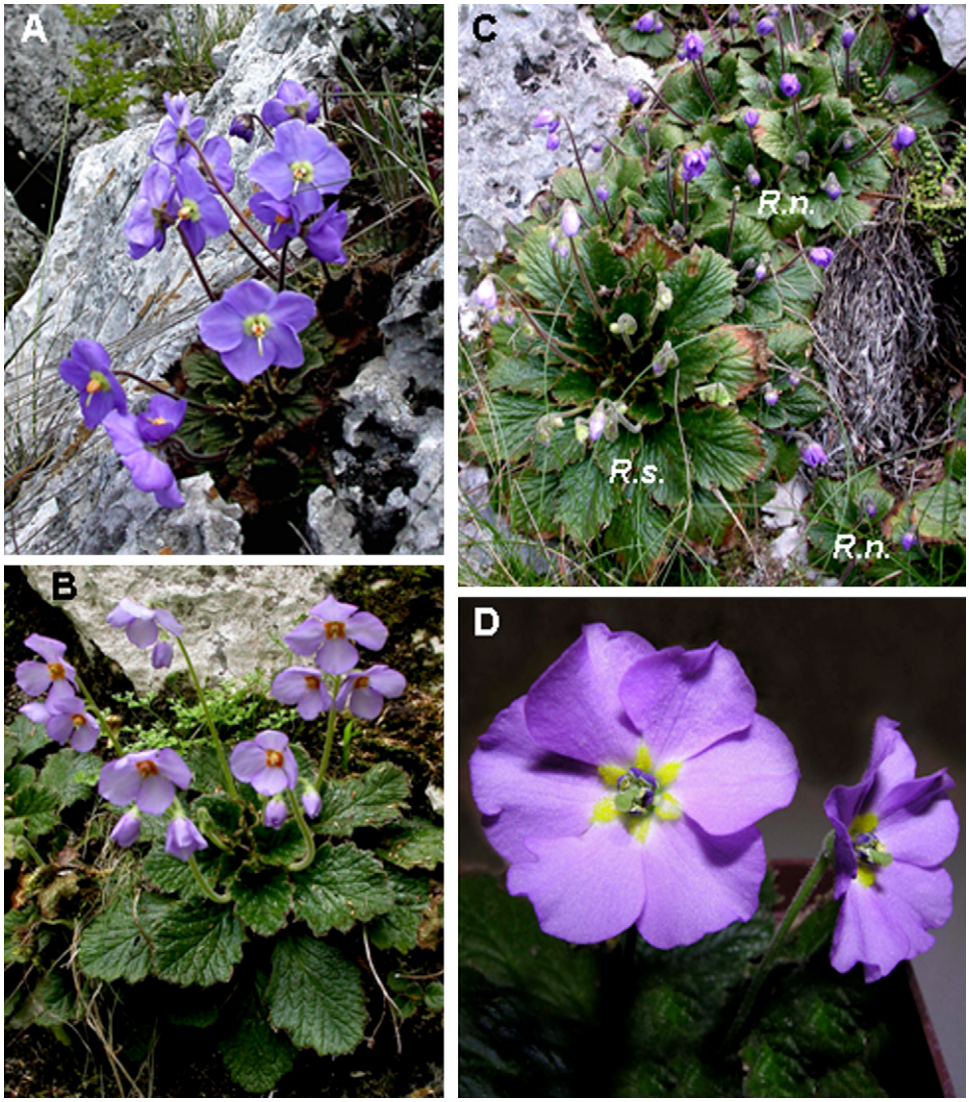

Fig. 2. *Ramonda nathaliae* from population Radovanski Kamen (A), *R. serbica* from population Oblik—Sicevacka Klisura (B), *R. nathaliae* (*R.n.*) and *R. serbica* (*R.s.*) in sympatric population Radovanski Kamen (C), high polyploid *R. serbica* from Cijevna population showing flowers with six and five petals (D). Authors of photographs: V. Stevanovic (A and B), B. Zlatkovic (C), S. Siljak-Yakovlev (D).

Aldrich) and, in separate analyses, GC-specific fluorochrome mithramycin (50 μg/mL). For mithramycin, an incubation of 20 min was necessary before measurement.

DNA content of 5000–10,000 stained nuclei was determined for each sample using an Elite ESP flow cytometer (Beckman-Coulter, Roissy, France) with a water-cooled argon laser. Total 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of the unknown specimen and the known internal standard. Base composition (GC percentage) was calculated using the nonlinear model established by Godelle et al. (1993). Each studied population comprised at least five individuals, measured separately and with repetition.

2.5. Statistical analysis

Data was analysed by Statistica 5.0, using one-way ANOVA test, followed by Tukey Highest Significant Difference (HSD) test. For *R. serbica* we used the Unequal N HSD test because of the unequal number of samples.

3. Results

Data on chromosome number and nuclear DNA content for 21 populations of the three investigated species are presented in Table 2. The base composition (GC%) of genus *Ramonda* was 42.1% GC for *R. nathaliae*, 39.9% for *R. myconi* and 41.2% for *R. serbica*. The mean 2C DNA value ranged from 2.30 for *R. nathaliae* to 2.59 pg for *R. myconi* and 7.91 pg for *R. serbica*.

Despite its fragmentary distribution and ecologically contrasting habitats (one population on serpentine substrate) there were no significant variations of DNA amount among six populations of *R. nathaliae* (mean values from 2.26 to 2.33 pg). On the contrary, significant differences in DNA amount were observed among eleven populations of *R. serbica* with mean values between 7.53 and 8.32 pg ($F = 11.06$, $p < 0.05$). Unequal N

Table 2
Chromosome numbers and nuclear DNA content in populations of three *Ramonda* species

Population code	Species	2*n*	Ploidy level (*x*)	2C in pg and (Mbp)[a]	*N*	S.D. (pg)	CV (%)	Minimum–maximum of 2C (pg)	Cx in pg and (Mbp)
1	*R. serbica*			7.58 (7413.24)	5	0.10	1.33	7.42–7.69	1.26 (1232.28)
2	*R. serbica*	144 ± 2	6	7.84 (7667.52)	4	0.08	0.97	7.74–7.91	1.31 (1277.92)
3	*R. serbica*	>144	6	8.24 (8058.72)	5	0.12	1.49	8.02–8.31	1.37 (1339.86)
4	*R. serbica*	144	6	7.53 (7364.34)	3	0.08	1.06	7.48–7.62	1.26 (1217.04)
5	*R. serbica*			8.08 (7902.24)	5	0.26	3.20	7.80–8.37	1.35 (1320.3)
6	*R. serbica*	144	6	8.13 (7951.14)	2	0.14	1.71	8.03–8.23	1.35 (1325.19)
7	*R. serbica*	144		8.32 (8136.96)	3	0.15	1.82	8.21–8.49	1.39 (1356.16)
8	*R. serbica*	144 ± 6 192 ∼230	6 8 10	7.82 (7647.96) (8.63)[b]	5	0.15 (1.79)[b]	1.92 (20.77)[b]	7.65–8.04 (11.82)[b]	1.30 (1274.66)
9	*R. serbica*	∼150	6	7.94 (7765.32)	5	0.19	2.36	7.72–8.13	1.32 (1294.22)
10	*R. serbica*	144	6	7.77 (7599.06)	5	0.04	0.48	7.71–7.79	1.30 (1266.51)
11	*R. serbica*			7.84 (7667.52)	2	0.17	2.22	7.71–7.96	1.31 (1277.92)
Mean for species				7.91 (7743.09)					1.32 (1292.17)
12	*R. nathaliae*	48	2	2.33 (2278.74)	5	0.04	1.90	2.28–2.37	1.17 (1139.37)
13	*R. nathaliae*			2.28 (2229.84)	5	0.05	2.34	2.22–2.35	1.14 (1114.92)
14	*R. nathaliae*	48	2	2.33 (2278.74)	5	0.04	1.70	2.27–2.36	1.17 (1139.37)
15	*R. nathaliae*			2.32 (2268.96)	5	0.02	0.94	2.30–2.35	1.16 (1134.48)
16	*R. nathaliae*			2.26 (2210.28)	5	0.01	0.56	2.25–2.28	1.13 (1105.14)
17	*R. nathaliae*			2.28 (2229.84)	5	0.04	1.93	2.23–2.34	1.14 (1114.92)
Mean for species				2.30 (2249.4)					1.15 (1124.7)
18	Sympatric populations Radovanski Kamen	96	4–8	6.83 (6679.74)	10	1.92	28.18	4.95–9.75	
19	Sympatric populations Oblik	96	4	5.20 (5085.6)	10	0.32	6.06	5.03–6.12	1.30 (1274.66)
20	*R. myconi*	48	2	2.53 (2474.34)	6	0.03	1.24	2.48–2.57	1.27 (1237.17)
21	*R. myconi*	48	2	2.66 (2601.48)	4	0.01	0.22	2.65–2.66	1.33 (1300.74)
Mean for species				2.59 (2537.91)					1.30 (1271.4)

N = number of plants.
[a] pg = 978 Mbp (according to Doležel et al., 2003).
[b] Mean value for population including one individual which showed the highest DNA value (11.82 pg).

HSD points out the marked differences among populations from the gorge of the River Nisava (population 3) and the Crna Reka (population 7) on the one hand, and populations of the canyon of River Lazareva Reka (population 1), Radovanski Kamen (population 4) and the gorge of stream Kroni e Besit (population 10) on the other hand. There were also significant differences between the population from the canyon of River Lazareva Reka (population 1) and population 5 from Oblik (Table 3).

Furthermore, significant intra-population variation of DNA amount was observed for *R. serbica* in one population from Montenegro (Cijevna). The 2C DNA content ranged from 2C = 7.65 to 11.82 pg and revealed existence of different ploidy levels among individuals of this population. In sympatric populations the values of 2C were predominantly intermediary (mean value 5.19 pg; Fig. 3A and B) between *R. nathaliae* (2.30 pg) and *R. serbica* (7.91 pg). The range of 2C DNA content in the sympatric population from Radovanski Kamen (from 4.95 to 9.75 pg; Fig. 3B) was larger than that in the Oblik population (from 5.3 to 6.12 pg; Fig. 3A) and their means were significantly different ($F = 6.65$, $p < 0.05$).

The chromosome number was determined for several populations from each species. In two populations of *R. myconi* and *R. nathaliae* the chromosome number was $2n = 2x = 48$ (Fig. 4C and D). The chromosome number of several *R. serbica* (Fig. 4E) populations showed this species to be hexaploid ($2n = 6x = 144$). Hyper- and hypoaneuploidy were also observed, coherent with the modest intra-population variation of 2C DNA value. Despite this variation in chromosome numbers, pollen grains showed uniform size and high viability (Fig. 4B). All populations of *R. serbica* investigated in this study had genome sizes corresponding to $6x$ ploidy level with $x = 24$ as basic chromosome

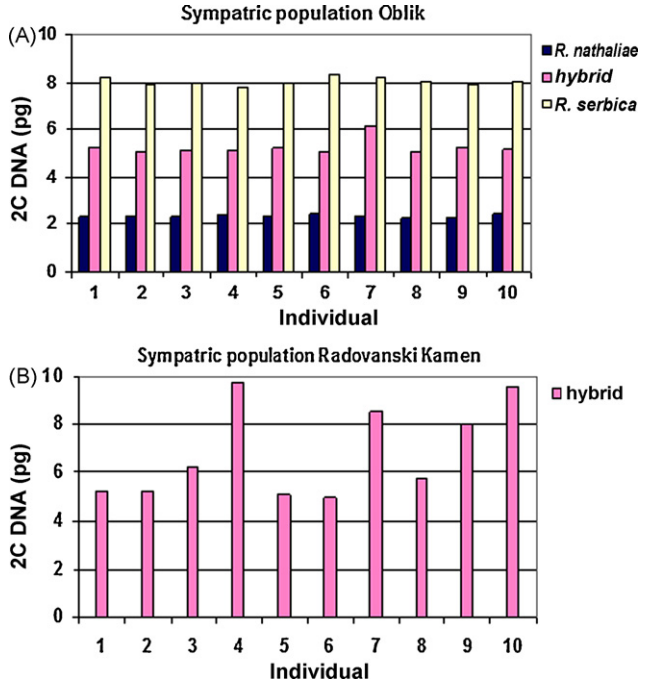

Fig. 3. Nuclear 2C DNA content for 10 individuals of *R. serbica*, *R. nathaliae* and their hybrid in sympatric population Oblik (A) and for hybrids in Radovanski Kamen sympatric population (B).

Table 3
Results of one-way ANOVA followed by unequal N HSD test for *R. serbica*

	Populations										
	{1} ($M = 7.5796$)	{2} ($M = 7.8440$)	{3} ($M = 8.2425$)	{4} ($M = 7.5309$)	{5} ($M = 8.0773$)	{6} ($M = 8.1288$)	{7} ($M = 8.3193$)	{8} ($M = 7.8198$)	{9} ($M = 7.9363$)	{10} ($M = 7.7705$)	{11} ($M = 7.8370$)
Lazareva Reka {1}		0.330391	0.000184**	0.999998	0.000494**	0.027558*	0.000207**	0.309958	0.021494*	0.629056	0.807871
Jelasnica gorge {2}	0.330391		0.021663*	0.297193	0.506341	0.701586	0.015770*	1.000000	0.997997	0.999715	1.000000
Nisava gorge {3}	0.000184**	0.021663*		0.000235**	0.794046	0.999383	0.999884	0.003429**	0.077807	0.000894**	0.229359
Radovanski Kamen {4}	0.999998	0.297193	0.000235**		0.003376**	0.011966*	0.000189**	0.406349	0.064367	0.665039	0.610229
Oblik {5}	0.000494**	0.506341	0.794046	0.003376**		1.000000	0.652365	0.224914	0.909072	0.076670	0.863117
Prizrenska Bistrica {6}	0.027558*	0.701586	0.999383	0.011966*	1.000000		0.965493	0.597365	0.962968	0.388644	0.671854
Crna Reka {7}	0.000207**	0.015770*	0.999884	0.000189**	0.652365	0.965493		0.009383**	0.097305	0.003205**	0.079933
Cijevna {8}	0.309958	1.000000	0.003429**	0.406349	0.224914	0.597365	0.009383**		0.972396	0.999979	1.000000
Kroni e Murici {9}	0.021494*	0.997997	0.077807	0.064367	0.909072	0.962968	0.097305	0.972396		0.790490	0.999811
Kroni e Besit {10}	0.629056	0.999715	0.000894**	0.665039	0.076670	0.388644	0.003205**	0.999979	0.790490		0.999995
Vikos {11}	0.807871	1.000000	0.229359	0.610229	0.863117	0.671854	0.079933	1.000000	0.999811	0.999995	

M, Mean value.
* Significant differences at $p < 0.05$.
** Significant differences at $p < 0.01$.

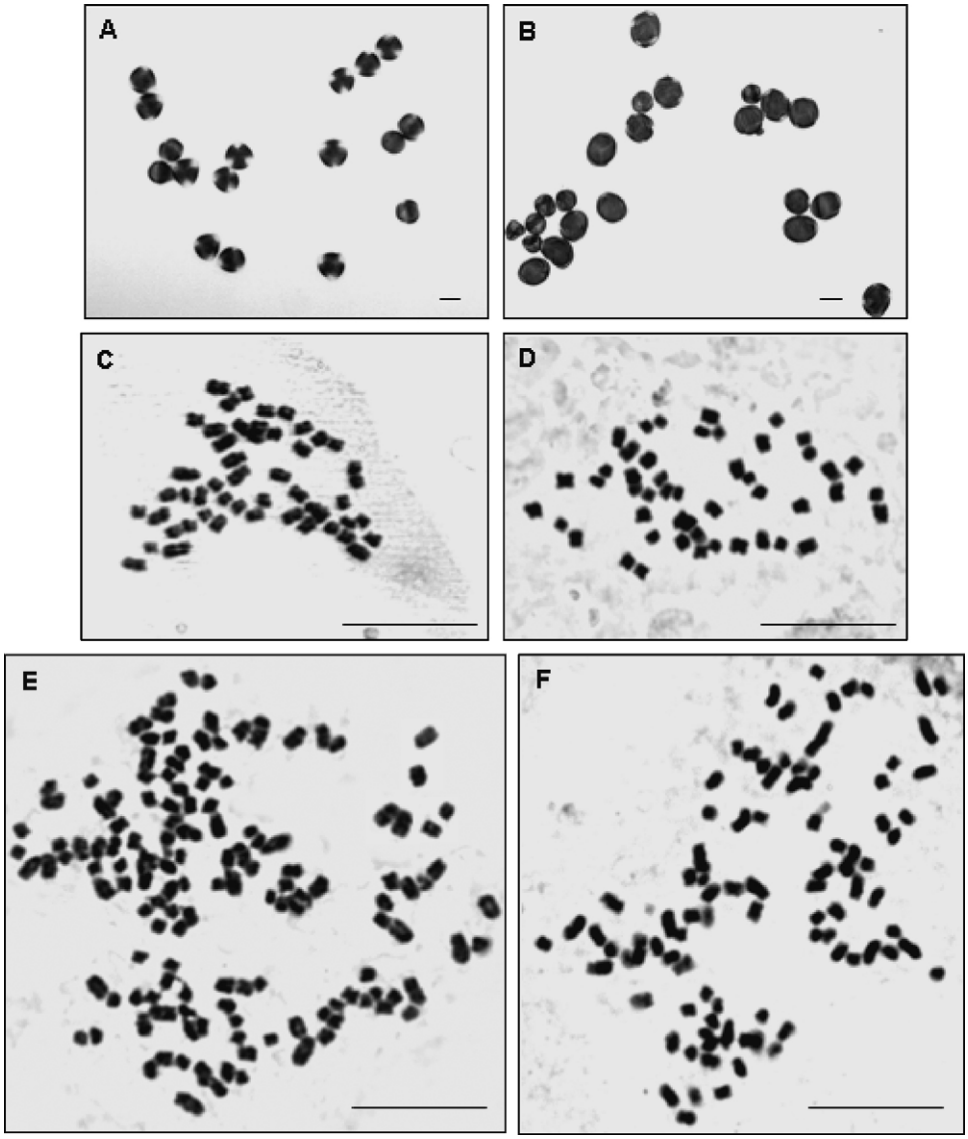

Fig. 4. Pollen of hybrid individual from sympatric population Oblik (A) and of *R. serbica* from the same population (B); metaphase plates of *R. myconi* (C) and *R. nathaliae* (D) with $2n = 48$ and *R. serbica* with $2n = 144$ (E); chromosomes of hybrid ($2n = 96$) from Oblik population (F). Bar = 10 μm.

number. Only one individual from Cijevna population with a higher 2C value (11.82 pg) and flowers with five or six petals (Fig. 2D) was probably decaploid ($2n = 10x = 240$). The majority of hybrid individuals from sympatric populations had about $2C \approx 5$ pg and $2n = 96$, but some specimens showed the higher values which could result from backcross with *R. serbica* or from spontaneous doubling of a hybrid genome (Table 2 and Figs. 3A and B and 4F). Pollen grains of hybrid individuals showed heterogeneity in size and low viability (Fig. 4A).

During our field investigations we established that the diploid species – smaller individuals with smaller genome size – are less tolerant of to air humidity stress and enter into anabiotic state earlier than the hexaploid species and the natural hybrids.

4. Discussion

In this study we report the first data on GC percentage for the three *Ramonda* species and nuclear DNA amount for *R. nathaliae* and *R. serbica*. Our samplings of *R. myconi* (2.59 pg) agree with the single value, 2.6 pg, reported by Zonneveld et al. (2005). *R. nathaliae* presents the smallest genome size (2.3 pg) but the highest GC percent (42.06%). The decrease of DNA content in this species is probably due to the loss of AT sequences.

A predominantly intermediary 2C value in sympatric populations indicates the hybridization ability between *R. serbica* and *R. nathaliae*. The hybrids were morphologically similar to *R. serbica.* This is expected since the genome of a hybrid between a hexaploid and a diploid contains three parts of one species (*R. serbica*) and one part of the other species (*R. nathaliae*). In addition, the pollen grains of hybrids show strong heterogeneity in size and low viability (Fig. 4A), while the pollen of typical populations of both species is homogeneous and highly viable (Fig. 4B).

In several *R. serbica* populations the chromosome number indicates this species to be hexaploid ($2n = 6x = 144$). However, these results are not in accordance with earlier observations

describing *R. serbica* as tetraploid with $2n = 72$ or $2n = 96$ (Glisic, 1924; Contandriopoulos, 1966, respectively). The chromosome number ($2n = 96$) found by Contandriopoulos (1966) probably corresponded to a hybrid individual, while $2n = 72$ found by Glisic (1924) could be that of one backcross between the hybrid ($2n = 96$) and *R. nathaliae* ($2n = 48$). In this study we did not find such individuals. However, our future investigations will be in particular focused on these two sympatric populations.

During the evolution of *Ramonda* genus, polyploidization and other chromosomal changes have certainly occurred. For most angiosperms, chromosomal duplication is followed by genome divergence and also by diploidization, resulting in diversity of chromosome number and DNA amount (Wendel, 2000). We presume that two species with $x = 24$ probably represent the ancient polyploids (paleopolyploids) which have been completely diploidized. The lowest basic chromosome number found for the family Gesneriaceae is $x = 4$ (*Chirita pumila*) and there is the panel of very different numbers $x = 8, 9, 11, 12, 13, 14, 15$ (Fedorov, 1969). The origin of high polyploidy in *R. serbica* could be explained by encounter of one reduced and one non-reduced gamete followed by duplication of chromosome set. In this case *R. serbica* could be considered as autopolyploid. The value of Cx (monoploid genome size according to Greilhuber et al., 2005) was essentially the same for *R. myconi* and *R. serbica* (1.30 and 1.32 pg, respectively) while it was 1.15 pg for *R. nathaliae*.

It is improbable that *R. serbica* differentiated from *R. nathaliae* because their Cx values are clearly different. However, *R serbica* and *R. myconi* did probably differentiate from a common ancestor (the same Cx values) before the geographical isolation of these two species. *R. myconi* then continued its evolution at the diploid (diploidized paleo-polyploid) level while *R. serbica* evolved through high polyploidisation. It is not excluded that tetraploid populations of *R. serbica* exist, but they have not yet been found. It is interesting that in *R. serbica*, the phenomenon of genome-downsizing described by Leitch and Bennett (2004), generally present in high polyploids, is not observed. In addition, a distinct Cx value for *R. nathaliae* in comparison to *R. serbica* and *R. myconi* suggests existence of a second ancestor which had survived in the Balkans, evolving into the recent *R. nathaliae*. Our on-going investigation of the phylogeography of populations of three *Ramonda* species should clarify their age and origin.

In the sympatric population of Oblik, the global mean was 1.30 pg, similar to the mean Cx value considering only the hybrid individuals, ~1.28 pg. Taking into account that hybrid genomes are potentially composed of three parts of *R. serbica* (Cx 1.32 pg) and one part of *R. nathaliae* genetic information (Cx 1.15 pg), the arithmetic mean of the hybrids would be 1.28 pg. In Radovanski Kamen (population 18) three types of individuals were observed: hybrid (~5 pg, with 96 chromosomes), backcross of hybrid with *R. serbica* (~6 pg) and hybrids which had doubled both in ploidy level (8×) and genome size (~9.5 pg). These latter two interpretations based only on genome size assessments will be verified by chromosome counts of such specimens.

We note that artificial hybridization between different European genera of Gesneriaceae for horticultural purposes is possible: Jancaemonda (*J. heldreichii* × *R. serbica*), Jancaeberlea (*J. heldreichii* × *Haberlea rhooprensis*), Ramberlea (*R. myconi* × *H. rhodopensis*) (Halda, 1989).

During the past decade the interest for resurrection flowering plants has increased, as they provide suitable model-systems for functional investigations concerning drought stress that might be relevant to improving crop plant resistance, and for identifying genes responsible for the drastic desiccation tolerance and the effective membrane protection mechanisms of plant survival under different environmental stresses (Dražić et al., 1999; Navari-Izzo and Rascio, 1999; Black and Pritchard, 2002; Proctor and Tuba, 2002; Bartels and Sunkar, 2005; Rascio and La Rocca, 2005). Our results provide insight into the evolutionary processes of these paleoendemic resurrection plants which are indeed preglacial relicts. Moreover, the results underline the need for cytogenetic characterisation of material, as polyploidy and natural hybridisation appear to be associated with enhanced resistance: is this due to specific genes, or a gene copy-number effect related to ploidy level or, indirectly, to a cell size effect?

4.1. Concluding remarks

These cytogenetic analyses of 21 populations of three *Ramonda* species have provided information relevant to understanding the evolutionary history of these rare resurrection plants of the Northern hemisphere. According to our investigations of nuclear DNA amounts and determination of chromosome number, it can be established that: (1) *R. nathaliae* and *R. myconi* are diploid species ($2n = 48$) with three times less DNA amount compared with the closely related hexaploid species *R. serbica* ($2n = 144$); (2) *R. myconi* and *R. serbica* have the smaller GC percentage and higher Cx value (39.9 and 41.2%; 1.32 and 1.30, respectively) while *R. nathaliae* has 42.1% of GC and Cx = 1.15; (3) the significant intrapopulation variations of 2C DNA content occurring in *R. serbic*a from one population from Montenegro (Cijevna, population 8) correspond to different ploidy levels within the same population; (4) hybridization occurs between *R. nathaliae* and *R. serbica* in sympatric zones and (5) polyploidy has been the major evolutionary mechanism in the genus *Ramonda*.

The examination of the origin and evolution of polyploidy into the complex of *Ramonda* species would be of particular interest from phylogenetic and phylogeographic considerations. There is a need to reconstruct the chorological history and cytogeography of these three paleoendemic species from the Balkan and Iberian Peninsulas. An investigation of many populations of each species is already underway to determine their degree of differentiation, exploiting morphological differences observed in certain parts of their range, particularly in sympatric and/or parapatric zones (Stevanovic et al., 1986a). The present results indicate that analyses should be broadened, involving more populations of all three species. Thereafter, it could be possible to develop a solid explanation of the interpopulation differentiation in general and of the intrapopulation variation of genome size and chromosome number.

Thus, a principal goal for our future investigations will be the determination of genome size and ploidy level in a more exten-

sive panel of populations, especially for the two Balkan species whose distribution ranges are very close. This way it would be possible to elucidate to what extent the Balkan *Ramonda* species remain microgeographically differentiated in overlapping zones, given that hybridization was only observed in two truly sympatric populations—a novel finding of the present study.

These cytogenetic and phytogeographic approaches, together with molecular investigations, should make clear the origin and phylogeographic relationship among three *Ramonda* species as well as the relations between the ancient flora of the Balkan and Iberian Peninsulas. Essentially, *Ramonda* offers a diversity of populations with exceptional resistance: clarifying their genetic relationships and the significance of ploidy changes should contribute to the larger question of understanding desiccation tolerance for agronomic situations.

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References

Alexander, M.P., 1969. Differential staining of aborted and non-aborted pollen. Stain Technol. 44, 117–122.

Bartels, D., Sunkar, R., 2005. Drought and salt tolerance in plants. Crit. Rev. Plant Sci. 23, 23–58.

Bennetzen, J.L., 2002. Mechanisms and rates of genome expansion and contraction in flowering plants. Genetica 115, 29–36.

Bennetzen, J.L., Ma, J., Devos, K.M., 2005. Mechanisms of recent genome size variation in flowering plants. Ann. Bot. 95, 127–132.

Black, M., Pritchard, H.W. (Eds.), 2002. Desiccation and Survival in Plants: Drying Without Dying. CABI Publishing, Wallingford, UK.

Contandriopoulos, J., 1966. Contribution à l'étude caryologique des Gésneriacées d'Europe et de leur germination. In: 91 Congrès des Sociétés Savantes, vol. 3, Rennes, pp. 271–280.

Bolos, O., Vigo, J., 1995. Flora dels Països Catalans, vol. III. Barcino, Barcelona.

Doležel, J., Bartoš, J., Voglmayr, H., Greilhuber, J., 2003. Nuclear DNA content and genome size of trout and human. Cytometry 51A, 127–128.

Dražić, G., Mihailović, N., Stevanović, B., 1999. Chlorophyll metabolism in leaves of higher Poikilohydric plants *Ramonda serbica* Panč. and *Ramonda nathaliae* Panč. et Petrov. during dehydration and rehydration. J. Plant Physiol. 154, 379–384.

Fedorov, A., 1969. Chromosome Number of Flowering Plants. Academy of Sciences of the USSR.

Feulgen, R., Rossenbeck, H., 1924. Mikroskopisch-chemischer Nachweis einer Nukleinsäure vom Typus der Thymonukleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. Hoppe-Seyler's Zeitschr. Physiol. Chem. 135, 203–248.

Galbraith, D., Harkins, K., Maddox, J., Ayres, N., Sharma, D., Firoozabady, E., 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220, 1049–1051.

Glisic, Lj., 1924. Development of the female x-generation and embryo in *Ramonda*. Ph.D. Thesis. University of Belgrade, Yugoslavia.

Godelle, B., Cartier, D., Marie, D., Brown, S.C., Siljak-Yakovlev, S., 1993. Heterochromatin study demonstrating the non-linearity of fluorometry useful for calculating genomic base composition. Cytometry 14, 618–626.

Gregory, T.R., 2005. The C-value enigma in plants and animals: a review of parallels and an appeal for partnership. Ann. Bot. 95, 133–146.

Greilhuber, J., Doležel, J., Lysàk, M.A., Bennett, M.D., 2005. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. Ann. Bot. 95, 255–260.

Halda, J., 1989. Four new intergeneric hybrids in the family Gesneriaceae. Preslia 61, 125–127.

Jakob, S.S., Meister, A., Blattner, R.F., 2004. The considerable genome size variation of *Hordeum* species (Poaceae) is linked to phylogeny, life form, ecology and speciation rates. Mol. Biol. Evol. 21, 860–869.

Jankovic, M., Stevanovic, V., 1981. Contribution to the study of *Ramonda serbica* Panc. phytocoenoses in the gorges of the Sara mountain northern slopes. Ekologija (Beograd) 16, 1–34.

Kosanin, N., 1921. La distribution géographique des deux espèces de *Ramondia* du Balkan. Academie des Sciences et Arts de Belgrade, 34–49.

Kosanin, N., 1939. Contribution to the biology of *Ramondia nathaliae*, *R. serbica* i *Ceterach officinarum*. Spomenik Srp. Kralj. Akad. LXXXIX, Prvi razred 20, 1–68 (in Serbian).

Kurtto, A., Lampinen, R., Junikka, L. (Eds.), 2004. Atlas Florae Europaeae. Distribution of Vascular Plants in Europe. 13 Rosaceae. The Committee for Mapping the Flora of Europe and Societas Biologica Fennica, Helsinki, pp. 14–15.

Leitch, I.J., Bennett, M.D., 2004. Genome downsizing in polyploid plants. Biol. J. Linnean Soc. 82, 651–663.

Marie, D., Brown, S., 1993. A cytometric exercise in plant DNA histograms, with 2C values for 70 species. Biol. Cell 78, 41–51.

Meyer, K.F., 1970. *Gesneriaceae* als Glieder der Flora des Tertiär in Europa. Wiss. Ztschr. Friedrich-Schiller Univ. Jena. Math. Naturwiss. Reiche 19 (3), 401–411.

Micevski, K., 1956. Eine uberprufung der verbreitungsgebite von *Ramonda nathaliae* Panc. et Petrov. und *Ramonda serbica* Panc. in Mazedonien und eine zusammenfassung der kakarteristischen merkmalen der beiden Arten. Annuaire de la Faculté de Philosophie de l'Université de Skopje 9, 121–142.

Navari-Izzo, F., Rascio, N., 1999. Plant response to water-deficit conditions. In: Pessarakli, M. (Ed.), Handbook of Plant and Crop Stress. Marcel Dekker, New York, NY, pp. 231–270.

Pancic, J., 1874. Flora of principality of Serbia. Beograd (in Serbian).

Petrovic, S., 1885. Ramondas in Serbia. Glasnik srpskog učenog društva (Beograd) 62, 101–123 (in Serbian).

Proctor, M.C.F., Tuba, Z., 2002. Poikilohydry and homoihydry: anthithesis or spectrum of possibilities? New Phytologist 156, 327–349.

Quezel, P., 1968. Signification phytosociologique des Gésneriacées Grecques. Collectanea Botanica 7, 947–973.

Rascio, N., La Rocca, N., 2005. Resurrection plants: the puzzle of surviving extreme vegetative desiccation. Crit. Rev. Plant Sci. 24, 209–225.

Ratter, J.A., 1963. Some chromosome numbers in the *Gesneriaceae*. Notes Roy. Bot. Gard. Edinburgh 24, 221–229.

Stefanoff, B., Georgiev, T., 1937. *Ramondia serbica* Panc. in Bulgaria. Annuaire de l'Université de Sofia 15 (2), 42–53.

Stevanovic, B., 1986. Ecophysiological characteristics of the species *Ramonda serbica* Panc. and *R. nathaliae* Panc. et Petrov. Ekologija (Beograd) 21, 119–134.

Stevanovic, B., 1989. Water relations of *Ramonda serbica* Panc. and *R. nathaliae* Panc. et Petrov. in different habitat conditions. Glasnik Instituta za Botaniku Univerziteta u Beogradu 23, 47–55.

Stevanovic, B., Glisic, O., 1997. Eco-anatomical differences between Balkan endemo-relict species of *Gesneriaceae*. Bocconea 5, 661–666.

Stevanovic, V., Stevanovic, B., 1985. *Asplenio cuneifolii–Ramondaetun nathaliae*—new chasmophytic community on serpentine rocks in Macedonia. Glasnik Prirodnjackog Muzeja u Beogradu Ser. B 40, 75–87.

Stevanovic, V., Niketic, M., Stevanovic, B., 1986a. On distribution of endemic and relic species *Ramonda serbica* Panc. u SR Macedonia. Glasnik Prirodnjackog Muzeja u Beogradu Ser. B 41, 89–95.

Stevanovic, V., Niketic, M., Stevanovic, B., 1986b. Sympatric area of the sibling and endemo-relic species *Ramonda serbica* Panc. and *R. nathaliae* Panc. et

Petrov. (*Gesneriaceae*) in southeast Serbia (Yugoslavia). Glasnik Instituta za Botaniku Univerziteta u Beogradu 20, 45–54.

Stevanovic, V., Niketic, M., Stevanovic, B., 1987. Phytocoenological characteristics of sympatric habitats of endemo-relic species *Ramonda serbica* Panc. and *R. nathaliae* Panc. et Petrov. Glasnik Instituta za Botaniku Univerziteta u Beogradu 21, 17–26.

Stevanovic, V., Niketic, M., Stevanovic, B., 1991. Chorological differentiation of endemo-relic species *Ramonda serbica* Panc. and *R. nathaliae* Panc. et Petrov. (*Gesneriaceae*) on the Balkan peninsula. Botanica Chronica 10, 507–515.

Stevanovic, B., Pham Thi, A.T., Monteiro da Paula, F., Viera da Silva, J., 1992. Effect of dehydration and rehydration on the polar lipid and fatty acid composition of *Ramonda* species. Can. J. Bot. 70, 107–113.

Stevanovic, V., Tan, K., Petrova, A., 2004. Mapping the endemic flora of the Balkans—a progress report. In: XI Optima Meeting, p. 63 (Abstracts).

Wendel, J.F., 2000. Genome evolution in polyploids. Plant Mol. Biol. 42, 225–249.

Zonneveld, B.J.M., Leitch, I.J., Bennett, M.D., 2005. First nuclear DNA amounts in more than 300 angiosperms. Ann. Bot. 96, 229–244.