

REPRODUCTIVE TRAITS IN TWO ALPINE ENDEMIC SPECIES OF CAMPANULACEAE

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Received on March 7, 2024

Presented by D. Ivanov, Corresponding Member of BAS, on March 26, 2024

Abstract

Alpine plants are well adapted to environments with extreme conditions – low temperatures, a short growing season, increased air humidity and precipitation, and are therefore of particular interest for research. The aim of the present study is to reveal the main reproductive characteristics of two endemic species of the family Campanulaceae, namely *Campanula orbelica* (Balkan endemic) and *Jasione bulgarica* (Bulgarian endemic). The main features of the reproductive biology (embryological features, type of reproduction) were established based on the study of micro- and macrosporogenesis and the development of male and female gametophytes. The established features of the reproductive structures and processes characterized *C. orbelica* and *J. bulgarica* as sexually reproducing species. Despite the extreme conditions of existence, the studied species presented normal generative progresses leading to the formation of pollen and embryo, which ensures their successful reproduction, populations stability and their distribution in the subalpine environment.

In addition, the genome size of *C. orbelica* was studied by flow cytometry. The estimated genome size varied in the range $1C = 1.34\text{--}1.38$ pg. No deviation in the ploidy level was found in 22 specimens fresh leaves of which were collected from different parts of the Rila and Pirin Mts.

Key words: *Campanula*, embryology, genome size, *Jasione*

Financial support of the Bulgarian National Science Fund under project GLORIA-Bulgaria, Contract KP-06-H21/16 of 19.12.2018 is gratefully acknowledged.

<https://doi.org/10.7546/CRABS.2024.05.05>

Introduction. In 2019, a project was launched to study the impact of climate change on alpine plant diversity in Bulgaria. For this purpose, four permanent monitoring sites were installed in the Rila Mts as a part of the GLORIA global network. The alpine habitats are among those habitats where pollinators are sparse or uncertain, and are reservoirs of relict biodiversity with a large number of rare and endemic plants [1]. The plants growing in these habitats are subjected to harsh environmental pressures and ecological constraints (e.g., moisture shortage, thin soil, poor organic matter, and high alkaline soil), extreme and harsh climate (e.g. low temperature, high solar radiation, and strong wind), which may result in stronger pressure on pollination and seed setting [1]. The reproductive success of alpine plants can be reduced by uncertain pollinator service and low pollen output [2]. Studies of reproductive characteristics can improve our understanding of the evolutionary process and survival strategies of these plants.

The present work shows the results of the study on reproductive biology and genome size of two endemic alpine plant species from Campanulaceae family which often occur in and around the Bulgarian GLORIA monitoring sites: *Campanula orbelica* Pančić ($\equiv$ *C. alpina* subsp. *orbelica* (Pančić) Urum.) and *Jasione bulgarica* Stoj. & Stef.

Campanula orbelica occurs in most of the high mountains in Bulgaria on siliceous bedrock usually above 1700 m a.s.l. in the following floristic regions: Balkan Range (Western, Central), Vitosha Region (Vitosha Mt.), West Frontier Mts (Osogovo Mt.), Pirin Mts, Rila Mts, and Rhodopi Mts (Western, Central) [3]. It is a Balkan endemic, distributed in northern parts of the Balkan Peninsula with diploid chromosome number $2n=34$ [4, sub *C. alpina*].

Jasione bulgarica grows in subalpine grassland and open scrub communities, usually on siliceous bedrock, in the Balkan Range (Central), Vitosha Region (Vitosha Mt.), West Frontier Mts (Osogovo Mt.), Pirin Mts, Rila Mts, and the Rhodopi Mts (Western, Central), usually above 1700 m a.s.l. It is a Bulgarian endemic [3] with diploid chromosome number $2n=12$ [4].

Studies on the reproductive biology of representatives of the genera *Campanula* and *Jasione* are limited. In the genus *Campanula*, flowering, pollination and reproduction features have been investigated [5–10]. A few species have been studied embryologically, e.g. *C. americana* [11], *C. kachethica* [12], *C. kemulariae* [13], *C. sibirica*, *C. taurica* and *C. talievii* [14]. In *Jasione montana*, some peculiarities of egg apparatus were described [15]. The embryological structures and processes of two target endemic species have not been studied so far.

Materials and methods. The plant material used for the study was collected from wild populations of the two target species from the Rila and Pirin Mts (Table 1).

To estimate the reproductive capacity, features of the reproductive sphere and the pollen viability were studied. To reveal the features of structures and processes in male and female generative sphere, flower buds in different develop-

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Localities of the studied populations of *Campanula alpina* and *Jasione bulgarica*

Locality number	Locality description
20-41a	Bulgaria, Rila Mts, subalpine pasture W-SW of Golyam Kalin Peak, ca. 2510 m a.s.l., 42.17405 ° N, 23.26772 ° E, 22.07.2020, coll. V. Vladimirov
20-67	Bulgaria, Rila Mts, subalpine pasture NW of Deno Peak, along the tourist trail from Musala Hut to Chakar Voyvoda Hut, ca. 2500 m a.s.l., 42.20384 ° N, 23.59926 ° E, 16.09.2020, coll. V. Vladimirov
20-91	Bulgaria, Rila Mts, subalpine rocky grassland, Vazov (Damga) Peak, ca. 2650 m a.s.l., 42.18742 ° N, 23.31804 ° E, 10.10.2020, coll. V. Vladimirov
20-92	Bulgaria, Rila Mts, subalpine pastures, a peak NW of Babreka Lake, ca. 2480 m a.s.l., 42.20717 ° N, 23.30292 ° E, 10.10.2020, coll. V. Vladimirov
s.n.	Bulgaria, Pirin Mts, NW of Muratovo Lake, 2240 m a.s.l., 41.74713 ° N, 23.40402 ° E, October 2022, coll. S. Stoyanov

mental stages were fixed in the field (Rila Mts, locality no. 20-41a) in FAA (5 parts formalin: 5 parts glacial acetic acid: 90 parts 70% ethanol) and consecutively treated according to the classical paraffin methods: embedded in paraffin; cut with rotary microtome into 8–20 µm slices; stained with Heidenhain's haematoxylin; embedded in Entellan and mounted on permanent slides. Observations were carried out using light microscope "Olympus CX21". Microphotographs were taken with "Infinity lite" 1,4Mpx Digital camera. Pollen viability was evaluated by counting the pollen grains in thirty mature anthers on a visual field at magnification 100×.

To estimate the nuclear DNA content CyFlow SL Green flow cytometer (PARTEC, Germany), equipped with a green (532 nm) solid-state laser was used. Fresh leaves from plants of the target species were used for the study. *Pisum sativum* 'Kleine Rheinländerin' (1C = 4.38 pg) [16] was applied as an internal standard. The plant material was treated with the extraction and staining kit 'CyStain®PI Absolute P' (SYSMEX) following the protocol prescribed by the manufacturer. Altogether 5000 PI-stained particles were measured per run, with five runs per preparation. Runs with a CV above 5% for the studied species or the standard were discarded. For detecting any variation in the genome size in *C. orbelica*, three preparations, each with 5–7 specimens of the species + the standard, were prepared. In this case, only one run per preparation was measured, with 5000 PI-stained particles per run.

Results and discussion. The main features of the reproductive biology (embryological features, type of reproduction) were established on the basis of

study of micro- and macrosporogenesis and development of the male and female gametophytes.

Male generative sphere. The anther was tetrasporangiate (Fig. 1-A) with four-layered anther wall that developed centrifugally, according to the Dicotyledonous-type and consisted of: an epidermis, endothecium, a middle layer and a tapetum. At the beginning of anther's ontogenesis, the layers of the anther wall were similar in size and shape to their constituent cells but during meiosis in the microspore mother cells (MMCs) they undergo changes which lead to their clear distinction. The epidermis comprised one row of almost rectangular tangentially elongated cells. The cells of the endothecium extended radially and developed fibrous thickenings after the formation of one-nucleated pollen (Fig. 1-B, 1A-B). At the end of the meiosis in MMCs of *C. orbelica*, the cells of endothecium undergo mitosis in radial direction forming second endothecium layer. The formation of an additional layer in anther wall was reported in other *Campanula* species too (*C. kachethica* [12]; *C. kemulariae* [13]), but its origin was interpreted differently. GABEDAVA [13] described it as middle layer and MELIA and BARBLISHVILI [12] as endothecium. The cited additional layer we determine as endothecium given the observed on it fibrous thickenings. Similar development of the anther wall with two endothecium layers was observed and described for some species from the genus *Campanula* distributed in Crimea [14]. The middle layer was ephemeral. Its cells become flattened, pressed from the growing cells of the endothecium and tapetum, and degenerated about the second (homotypic) division of the meiosis. The tapetum was glandular and remained such up to the end of its existence (stage of formation of microspore tetrads after which began to degenerate). At the end of heterotypic division of meiosis in MMCs its cells become two-nucleate as a result of a mitotic divisions. The wall of mature anther consisted of persistent epidermis, fibrous endothecium and degenerating areas of the middle layer (Fig. 1-D, 1A-B). Meiosis in MMCs proceeded normally. Microspore tetrads formed by meiosis with simultaneous cytokinesis and are mostly tetrahedral. The mature pollen grains were 3-porous, bicellular (Fig. 1-C, 1A-C) that is the usual development of pollen in Campanulaceae described in several species of the family [17]. The mature pollen was morphologically uniform (Fig. 1-C, D, 1A-C). The estimated pollen viability was high in the studied species: 90% of examined mature pollen grains of *C. orbelica* and 93.3 % of these of *J. bulgarica* were fertile.

Our observations during the study showed that the two studied species are proterandrous. Like in other *Campanula* species (*C. armazica* [10]) the maturation of male and female spheres is segregated in time. This is a sign that the cross-pollination is the priority way of pollination for these species.

Female generative sphere. Ovary was observed to be inferior, three-locular, with numerous ovules in each locule (Fig. 2-A, 2A-A, B). The ovules were small, anatropous, with a very short funiculus (Fig. 2-B, 2A-C). The female gametophyte followed *Polygonum*-type of development like other species of

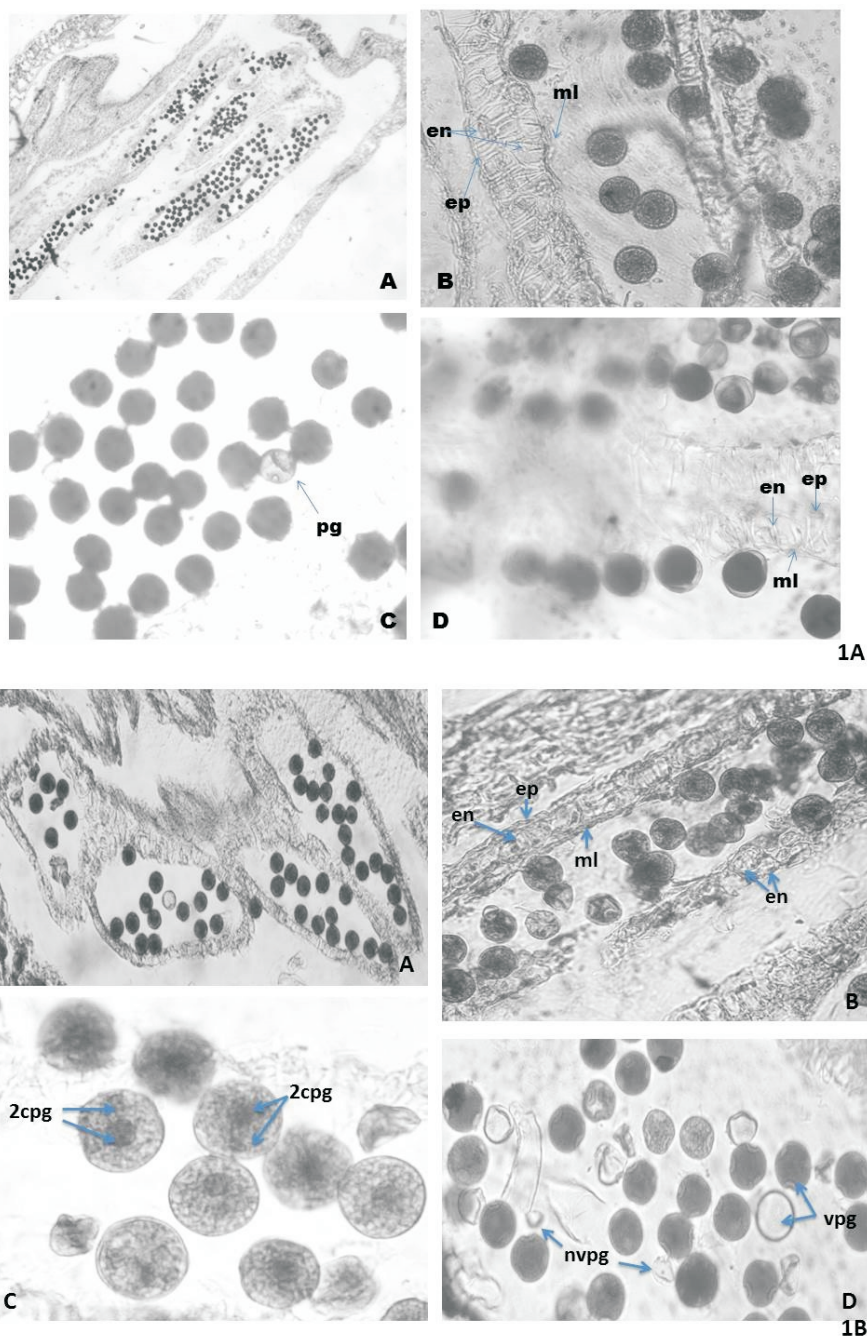


Fig. 1. Anther and development of the male gametophyte, **1A.** *Campanula orbelica*: A. – Tetrastrobangiate anther; B. – One-celled pollen and anther wall; C – Mature pollen grains; D. – Mature pollen and anther wall; **1B.** *Jasione bulgarica*: A. – Tetrastrobangiate anther; B. – One-celled pollen and anther wall; C – Two-celled mature pollen grains; D. – Viable and non-viable mature pollen and anther wall; *ep* – epidermis, *en* – endothecium, *ml* – middle layer, *pg* – pollen grain, *2cpg* – two-celled pollen grain

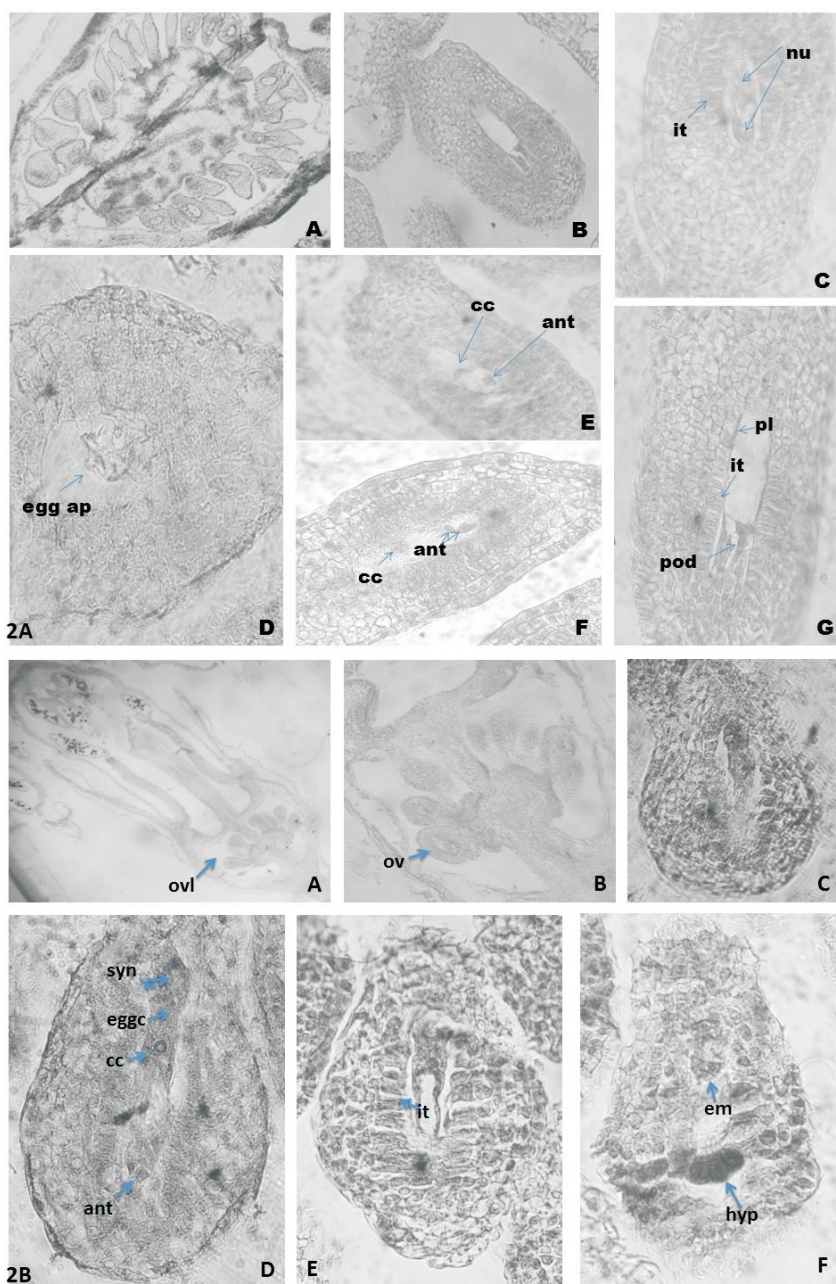


Fig. 2. Ovule and development of the female gametophyte: **2A.** *Campanula orbelica*: A – Ovary with ovules; B. – Anatropous ovule; C. – 2-nuclear ES; D. – Egg apparatus in the ES cavity; E, F – Central cell and antipoda in the ES cavity; G – ES with integumentary tapetum and podium; **2B.** *Jasione bulgarica*: A – Flower with anthers and ovary; B. – Ovary with ovules; C. – Anatropous ovule; D. – *Polygonum*-type ES with egg apparatus central cell and antipodals; E – Integumentary tapetum in the ES; F – Solanad-type embryo; it – integumentary tapetum, nu – nucleus, egg ap – egg apparatus, cc – central cell, ant – antipodia, pod – podium, pl – protoplasm layer, hyp – hypostase

Campanulaceae [17]. The cells of egg apparatus were small, with the typical arrangement and form (Fig. 2-D, 2A-D). Polar nuclei fused before the fertilization and the formed central cell is located near the egg cell (Fig. 2-E, 2A-D) or the wall of the embryo sac (ES) (Fig. 2-F). In the case when the central cell is located below the egg cell, it is in the form of a cross-drawn line. Similar view of the central cell in the region of the egg cell was described [17] as typical for the representatives of Campanulaceae and was named “maze”, interpreted as structure ensuring the course of intensive exchange processes in this area of ES. From the specialized structures of the ovule, described in the embryological literature [18], in the ovule of *C. orbelica* and *J. bulgarica* we observed the presence of integumentary tapetum, podium (Fig. 2-G) in *C. orbelica*, and hypostase (Fig. 2A-F) in *J. bulgarica*. The integumentary tapetum formed at stage of two-nucleate ES from the cells of the innermost layer of integument and surrounded the ES (Fig. 2-C, 2A-E). The tapetal cells are unusually large and appear, in longitudinal section, columnar (Fig. 2-G.). The same structure of integumentary tapetum was described also by BARNES [11] in *C. americana*. The podium (a cup-like structure) is the preserved part of the nucellus in the chalazal region of ES (Fig. 2-G). BATYGINA [18] distinguished different types of podium on the basis of the transformation pattern of this nucellar region: cup-shaped (differentiates as a cup-shaped structure from the persistent lateral region of the nucellus); columnar (the entire chalazal region of the nucellus transforms into the podium); postamento-podium (combining features of the postament and podium and comprising the entire chalazal region of the nucellus). The observed in the present study podium in *C. orbelica* we define as postamento-podium as the reported in other *Campanula* species (*C. sibirica*, *C. taurica*, *C. talievii* [18]). The hypostase is usually formed by disc-like cluster of cells or 1 to 3 thick-walled, densely cytoplasmic cells located immediately below the ES [19]. This structure is also reported for Campanulaceae [18]. The two structures cited perform similar functions associated with the transport of metabolites to gametophytic structures, support the water balance and protection against unfavourable environmental factors.

In the basal enlargement of the ES lie the three antipodal cells (Fig. 2-E, F, 2A-D). In *C. orbelica* a layer of protoplasm forms in the embryo sac between the egg-apparatus and the antipodal cells (Fig. 2-G). The same structure was described by Barnes [11] in *C. americana*. The endosperm was cellular, but initially goes through a free-nuclear phase: the primary cell of the endosperm divided transversally and formed two cells (micropylar and chalazal). The chalazal cell divided longitudinally, and its derivatives transversely, forming 2-celled layers. Subsequently, the cells of these two layers divided 1–2 times and more, also transversally. In *C. orbelica*, the last two cells of the lower endosperm layer differentiated into chalazal haustorium (Fig. 3-A, C). Such development of endosperm was described as characteristic of the representatives of the tribe *Campanuloideae* and genus *Campanula* [17]. KOROBOVA and ZHINKINA [17] determine the type

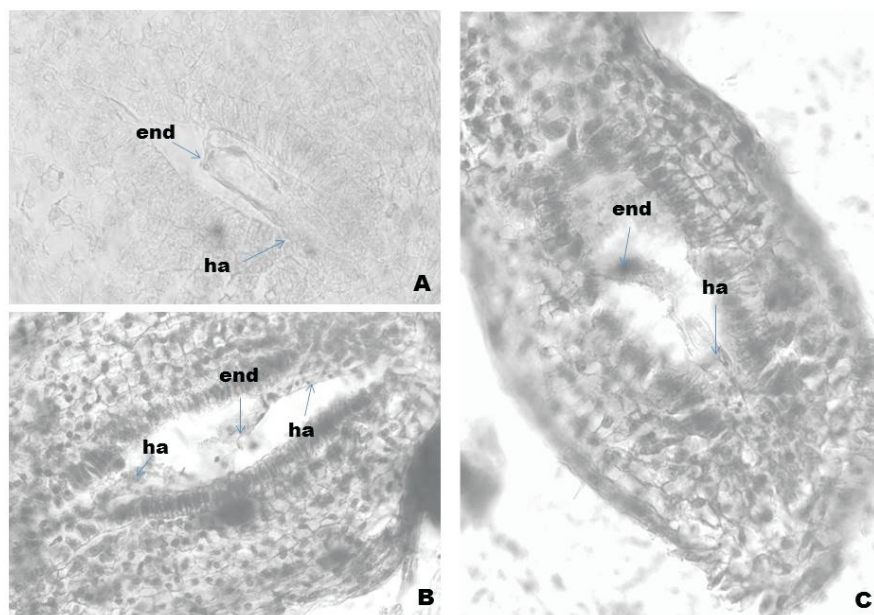


Fig. 3. Formation of endosperm haustorium. A, B – at the chalazal end; C – at both ends; ha – haustorium, end – endosperm nucleus

of endosperm development in *Campanula* species as *Codonopsis*-type. During the study we observed formation of haustorium on the micropilar end of endosperm (Fig. 3-B) despite the above described one in the chalazal end. The formation of endosperm haustoria (micropylar and chalazal) in Campanulaceae were also reported [19].

Embryogenesis is Solanad-type development that is typical for the family Campanulaceae [17].

Genome size. The nuclear DNA-amount (C-value) was measured in 22 specimens of *C. orbelica* (Table 2). The estimated genome size varies in the range $1C = 1.34\text{--}1.38$ pg. No variation in the ploidy level was detected which is congruent with the published chromosome counts of the species from Bulgarian accessions [4]. RONI-KIER and ZALEWSKA-GAŁO-SZ [20] in their flow cytometry studies of numerous samples of *C. alpina* s.l., in particular *C. alpina* and *C. orbelica*, from different countries and localities (including the Rila Mts) found that both species have similar genome size values, respectively, the same ploidy level.

Conclusions. As a result of the first study on the reproductive biology of *Campanula orbelica* and *Jasione bulgarica* the peculiarities of structure and processes in male and female generative sphere of the two endemic species from

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DNA content in *Campanula orbelica* from Bulgarian accessions, with *Pisum sativum* 'Kleine Rheinländerin' (1C = 4.38 pg) used as an internal standard

Locality	No of specimens measured	1C [pg]	CV [%; 5 runs]
Rila Mts, loc. 20-67	1	1.35	0.25
Rila Mts, loc. 20-67	5	1.37	–
Rila Mts, loc. 20-91	1	1.38	0.43
Rila Mts, loc. 20-91	6	1.36	–
Rila Mts, loc. 20-92	1	1.38	0.23
Rila Mts, loc. 20-92	7	1.36	–
Pirin Mts, s.n.	1	1.34	0.12

Campanulaceae were revealed. The established peculiarities of the reproductive structures and processes characterized the two studied taxa as sexually reproducing species. Despite the extreme alpine environment, the normal running of the processes of pollen and embryo formation ensure successful reproduction and explain the stability of the populations and the common occurrence of both taxa in the monitoring plots. The presence of specialized structures in the generative organs such as hypostase, podium and endosperm haustorium, providing better nutrition to the young embryo, ensures high adaptability of plants to environmental conditions and stability of their populations.

The results of the conducted research show that the male and female generative spheres of *Campanula orbelica* and *Jasione bulgarica* develop normally without essential disturbances. High pollen fertility was established. The embryo sac, embryo and endosperm form according to the described for Campanulaceae pattern. The normal course of the processes leading to pollen and seed formation is in concordance with diploid chromosome number of the two studied species.

The normal running of reproductive processes together with the high amount of predominantly fertile pollen are a prerequisite for a high degree of realization of the reproductive potential and successful reproduction of the populations, which in turn is a guarantee for preserving their size and number. In addition, the genome size of *C. orbelica* was established. No deviations in the ploidy level were found in plants collected from different parts of the distribution range of the species.

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