DE GRUYTER OPEN

Acta Bot. Croat. 75 (1), 81–88, 2016
DOI: 10.1515/botcro-2016-0020

CODEN: ABCRA 25
ISSN 0365-0588
eISSN 1847-8476

Morphological variability of the Bulgarian endemic *Betonica bulgarica* Degen et Neič. (Lamiaceae) from Sinite Kamani Natural Park, Eastern Balkan Range

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Abstract – Four populations of *Betonica bulgarica* Degen et Neič. at Sinite Kamani Natural Park were morphologically tested. Intrapopulation and interpopulation variabilities were established. The relationship between morphological variability, number, area and ecological appurtenance of the studied populations were explored. The results demonstrated that the main source of phenotype variation is intrapopulation variability, mainly due to the age structure of populations. The most variable traits are height of stem and dimensions of leaves. The registered interpopulation variability was affected by the differences in altitude, soil type and differences in environmental conditions and soil properties. Indumentum and morphology of generative organs had taxonomic significance for distinguishing *B. bulgarica* from the other species in the genus, including the species that were morphologically most similar to it – *Betonica officinalis* L.

Keywords: *Betonica bulgarica*, morphology populations, variations

Introduction

Endemic plants are an emblematic symbol of the Bulgarian flora and one of the most sensitive and vulnerable units in the country's nature ecosystems (Anchev 2011). They comprise 12.8% of the members of the flora of Bulgaria and demonstrate the specificity and genetic features of its flora (Petrova et al. 2005). The Bulgarian endemic *Betonica bulgarica* Degen et Neič. (syn. *Stachys bulgarica* Hayek), described by the Hungarian botanist A. V. Degen and the Bulgarian botanist I. Neichev in 1906 from materials collected in the Central Balkan Range, grows on open, stony and sandy ground in the oak and beech forest belts in the Balkan Range (Central, Eastern) and the Thracian Lowland (Velchev et al. 1992, Petrova 2006, Genova 2011). The species is morphologically similar to *Betonica officinalis* L. (syn. *Stachys officinalis* (L.) Trevis.). Although *B. bulgarica* is a protected species by the Biological Diversity Act of Bulgaria (Anonymous 2002) and most of its populations are found in protected territories – the Central Balkan National Park, Sinite Kamani Natural Park and in the protected areas of Natura 2000 – recent expert evaluations define it as endangered (Genova 2011). To this day, the species has not been subjected to detailed morphological studies. The polyphenol content in roots and above-ground parts has been studied (Bankova et al. 1999). Data about the state of *B. bulgarica* populations on the territory of Balkan Range are contained in the management plans of the Central Balkan National Park (Yankov et al. 2001) and Sinite Kamani Natural Park (Grozeva et al. 2004).

B. bulgarica was first registered in the Eastern Balkan Range by Neichev (1906) but materials were not deposited in Bulgarian scientific herbaria (SOM, SO, SOA). Nowadays the species locality specified by Neichev is part of the territory of the Sinite Kamani Natural Park. Grozeva et al. (2004) registered its population in the Ablanovo area of the park. Currently, the species populations have been registered in three other areas of the park territory – near the Microyazovir in Karandila area, on Slancheva Polyana and near Upper Lift Station (Grozeva et al. 2014). Their state was assessed and their requirements as to soil fertility were studied (Grozeva et al. 2014).

The objective of the present study was to establish the morphological population variability of *B. bulgarica* on the territory of Sinite Kamani Natural Park, to identify the traits that have the greatest taxonomic value and to seek the rela-

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tionship between the degree of morphological variability, area, number and ecological conditions of the studied populations.

Materials and methods

The subject of this survey were the four currently known populations of *B. bulgarica* in the territory of Sinite Kamani Natural Park (Tab. 1). Observations were carried out during the vegetation periods of 2013 and 2014, and morphometric tests were performed during the vegetation period of 2014. In each population the sites for taking morphological measurements were selected at the beginning of vegetation according to the presence of sufficient population density and the presence of *B. bulgarica* and at least four sites were identified. They were found along the diagonals in both directions of the population and areas in the central parts were deliberately included. The number of plants measured at each point was at least ten with a total of 50 plants used from each population. Morphometric measurements were conducted eight times during the vegetation period from April 5th to September 5th. The morphological analysis included 35 quantitative traits (On-line Suppl. Tabs. 1 and 2). A total of 21 qualitative traits were also analyzed (Tab. 2). Due to lack of variability in the studied 21 quantitative traits, the data obtained from the four investigated populations are presented in a summarized form in Tab. 2.

Flower and fruit dimensions, specifics of leaf epidermis and pollen morphology were recorded in laboratory conditions for flowers, fruit and leaves collected earlier from the population. Ripe fruits collected in isolation bags were measured. After the measurements, nutlets were sown within the borders of each population. All other characteristics were recorded directly on site. To study pollen and nutlet morphology and for a more detailed study of leaf epidermis and type of trichomes the electronic microscope method was used. Studies were conducted on dry plant parts in the laboratory at the Faculty of Chemistry and Pharmacy in St. Kliment Ohridski University of Sofia. At least twenty pollen grains and ten nutlets and leaves from each population were studied.

Pollen terminology followed Erdtman (1952) and Punt et al. (1994). Data from the microscopic measurements of pollen were presented in generalized form for the species due to lack of significant variability within and between populations.

The results obtained for the parameters related to the morphological characteristics were statistically processed with Statistica for Windows 10. The statistical reliability of differences among various parameters, the interpopulation and intrapopulation variations of traits were established through ANOVA. Cluster analysis was applied to group populations by similarity of 35 of the quantitative morphological traits. To allocate populations into groups by strength and direction of influence of the basic traits (from the included 35 morphological traits) principal component analysis (PCA) was conducted.

Results

The data on the mean arithmetic values of the studied quantitative traits and the variance values of each trait are presented in On-line Suppl. Tabs. 1 and 2. Interpopulation variability was traced based on variance values. The values differed both by individual traits of each population and among populations. The following traits were the most variable among all the populations: height of stem, basal leaf petiole length and stem leaf petiole length. In the populations from Ablanovo and Slancheva Polyana a higher am-

Tab. 1. Studied population of *Betonica bulgarica* Degen et Neič.

Locality	Ecological conditions	Population
Ablanovo area, N 42°42.638’, E 26°17.262’, 540 m a.s.l.	An open meadow on the edge of a mixed deciduous forest comprising *Carpinus betulus* L., *Quercus robur* L., *Ulmus minor* Mill., *Fraxinus ornus* L. and *Crataegus monogyna* Jacq. The grassland community is dominated by *Betonica bulgarica*.The terrain is very slightly sloped (3°–4°), non-eroded, facing south-east. The bedrock is limestone, the soil type – Chromic Luvisols (WRBSR, 2006).	The area of the population is 1600 m^2 and it numbers 440 specimens, incl. 310 flowering and 50 juvenile ones.
Upper lift station, N 42°43.100′, E 26°21.619′, 1015 m a.s.l.	An open meadow on the edge of a forest consisting of *Fagus sylvatica* L. ssp. *moesiaca* (K. Maly) Hjelmquist, *Picea abies* (L.) Karst. and *Pinus sylvestris* L. The grassland community is dominated by cereal species.The terrain has a slope of 7°, slightly eroded, dry, facing west. The bedrock is limestone, the soil type is Rendzinas (WRBSR, 2006). The plant community is dominated by cereal species.	The area of the population is 150 m^2 and it numbers 140 specimens, incl. 92 flowering and 48 juvenile ones.
Microyazovir area N 42°42.852′, E 26°22.654′, 945 m a.s.l.	An open meadow near the cliffs east of the dams. The terrain is very sloped 11°–20°, highly eroded, dry, facing north. The grassland community is dominated by *Sesleria latifolia* Degen.The bedrock is quartz porphyry, the soil type – Eutric Cambisols. Due to the reported high level of soil erosion in Karandila area, east of the dam, the soil in the studied area was determined as Regosols, too (WRBSR, 2006).	The population area is 950 m^2 and it numbers 95 specimens, incl. 74 flowering and 21 juvenile ones.
Slancheva polyana area N 42°43.252′, E 26°21.668′, 1001 m a.s.l.	An open meadow on the edge of a mixed forest consisting of *Fagus sylvatica* ssp. moesiaca, *Pinus silvestris*, *Fraxinus ornus*, *Prunus cerasifera* Ehrh., *Acer campestre* L., with some bushes of *Juniperus communis* L., *Crataegus monogyna*, *Rosa canina* L., *Rubus canescens* DC. In the grass layer *Festuca valesiaca* Schleich. ex Gaudin is dominant. The terrain has a slope of up to 11°, non-eroded, dry, facing west. The bedrock is limestone, the soil type – Eutric Cambisols.	The area of the population is 724 m^2 and it numbers 215 specimens, incl. 155 flowering and 60 juvenile ones.

Tab. 2. Studied qualitative features of *Betonica bulgarica* Degen et Neič.

	Feature	
1.	Colour of stem	Light green.
2.	Indumentum of stem	Non glandular multicellular, simple, uniseriate with micropapillae cultural surface trichomes; peltate glandular trichomes.
3.	Shape of leaf lamina	Rounded to elliptic and lanceolate, with a blunt tip, concave base and scalloped margin for annual plants developed from seeds; Lanceolate with a blunt tip, concave or asymmetric base and scalloped edge at the biennial and perennial plants developed from seeds and rhizomes.
4.	Colour of leaf lamina	Green to light green.
5.	Indumentum of leaf lamina	Adaxial and abaxial leaf surface – Non glandular multicellular, simple, uniseriate with micropapillae cultural surface trichomes; peltate glandular trichomes.
6.	Shape of epidermal cell of leaf lamina	Adaxial and abaxial leaf surface – elongated, polygonal, with varied size.
7.	Type of stomata	Abaxial leaf surface – anomocytic, diacytic, paracytic.
8.	Indumentum of leaf petiole	Non glandular multicellular, simple, uniseriate with micropapillae cultural surface trichomes; peltate glandular trichomes.
9.	Type of inflorescence	Flowers gathered in flower vertebrae, forming tight cyme. The bottom vertebra is often separated at a distance from the cluster.
10.	Shape of bracts	Lanceolate, with smooth edge, awned peak. Persists in the fruit.
11.	Indumentum of bracts	Adaxial and abaxial leaf surface – Non glandular multicellular, simple, uniseriate with micropapillae cultural surface trichomes along the edge and the primary vein; peltate glandular trichomes.
12.	Shape of calyx segments	Sepals fused in a tube, calyx teeth styliform, almost equal to the tube. Calyx retained in the fruit.
13.	Colour of calyx segments	Light green to dark purple.
14.	Indumentum of calyx segments	Calyx tube – Non glandular multicellular, simple, uniseriate with micropapillae cultural surface trichomes; non glandular stellate multicellular trichomes with 2–3 rays; peltate glandular trichomes. Calyx teeth – Non glandular multicellular, simple, uniseriate, hard, with smooth surface and peltate glandular trichomes.
15.	Shape of corolla segments	Corolla tube narrow, ± curved, upper lip entire, sometimes binary, lower lip ternary with wavy middle part and egg-shaped side parts.
16.	Colour of corolla segments	Light purple
17.	Indumentum of corolla segments	Non glandular multicellular, simple, uniseriate with micropapillae cultural surface trichomes; non glandular stellate multicellular trichomes with 2–3 rays; peltate glandular trichomes.
18.	Colour of anthers	Upon opening of the flower – yellow, during flowering – purple.
19.	Shape of nutlet	Triangular, elongated, the outside almost flat, the edges with narrow wings, which at the top edge go into irregularly toothed membranous appendage.
20.	Colour of nutlet	Dark brown
21.	Nutlet surface	Smooth formed by oblong cells variable in size

plitude of variability was registered for other traits as well: stem leaf petiole length, raceme and branch length.

The least variable in all the populations were the following traits: basal leaves length/width ratio, raceme leaves petiole length, first raceme leaves width, third raceme leaves width, corolla width base and those characterizing seed (length, width, length/width ratio). In the Ablanovo population a low level of variability was registered for other traits as well: corolla length/width top and calyx length, in the population at Slancheva Polyana – second raceme leaves petiole length, in the population at Upper Lift Station – raceme branch length and third raceme leaves length, and for that at Microyazovir – raceme branch length, second raceme leaves petiole length and calyx length/width ratio.

In the population at Slancheva Polyana no variability was found for third raceme leaves length and third raceme leaves length/width ratio or in the population at the Microyazovir for third raceme leaves length/width ratio.

The ANOVA results (On-line Suppl. Tabs. 1 and 2) showed that intrapopulation variability was dominant in the total variability. Higher interpopulation variability was found only for petiole length of basal leaves.

The data from the unweighted pairgroup average (UPGA) cluster analysis based on morphological pairwise similarities (Euclidean distances between population centroids) showed the greatest similarity in the entire complex of qualitative traits among specimens from the populations at Slancheva Polyana and Microyazovir (Fig. 1). The greatest differences were recorded for the population from the Ablanovo area.

Factor scores based on the principal component analysis of the distribution of populations according to the 35 morphological traits studied showed that the population at Ablanovo had positive values for both factor 1, and factor 2 (On-line Suppl. Tab. 3). The populations at Microyazovir and Slancheva Polyana had positive values for the second

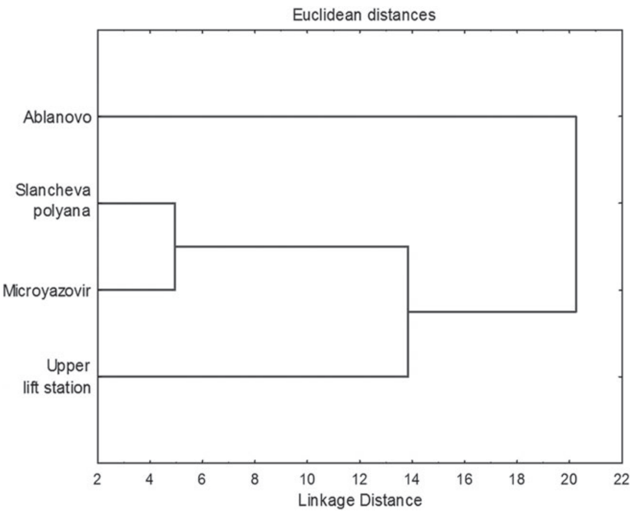

Fig. 1. Dendrogram of the cluster analysis of four *Betonica bulgarica* Degen et Neič. populations based on 35 traits of vegetative and generative morphological variability (see On-line Suppl. Tabs. 1 and 2).

factor but negative ones for the first one. The population at the Upper Lift Station area was negative for both factors. The population from Ablanovo exceeded the other populations by 11 out of a total of 35 studied traits. Height of plants, length, width of basal leaves, petiole length of basal leaves, length and width of stem leaves were significant.

The data related to the qualitative traits studied are presented in Tab. 2; Figs. 2, 3A–B and 4. In the four studied populations no significant variability in the types of trichomes and stomatas, pollens and nutlet morphology was established.

The epidermal surfaces of all examined plants showed both non-glandular and glandular trichomes (Tab. 2). Stem, leaves, bract, calyx and corolla were more or less covered with peltate glandular trichomes (Figs. 2 and 3A–B). On the fresh parts of *B. bulgarica* small capilate trichomes were found, practically invisible on the herbarised plant parts due to their small size. Stem, leaf petioles, bracts and the calyx tube were covered with non-glandular multicellular, simple, uniseriate trichomes with micropapillae cuticular surface (Figs. 2A–E and 3A). The calyx and corolla tube of *B. bulgarica* were covered with non-glandular stellate multicellular trichomes with two to three rays (Figs.

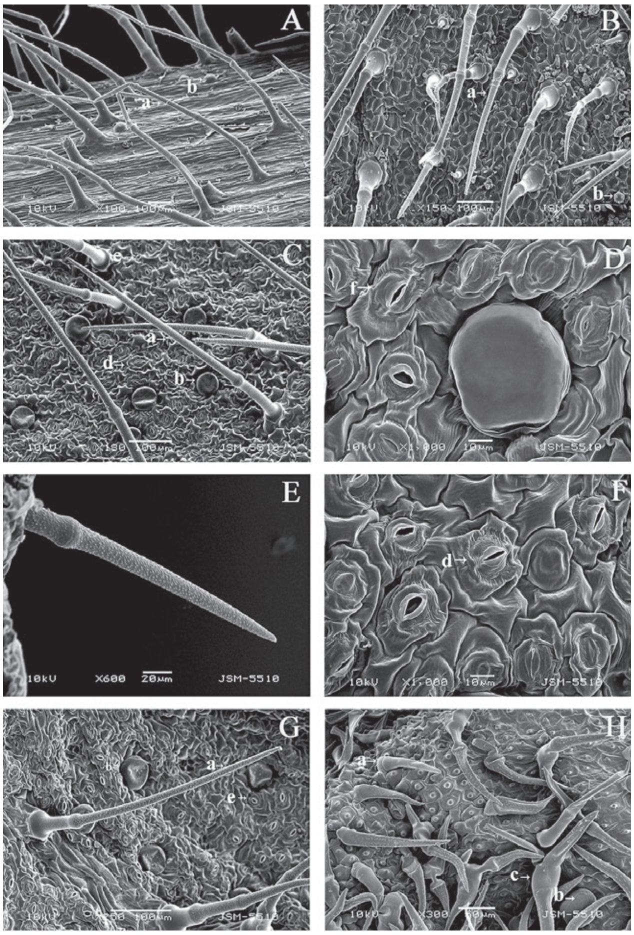

Fig. 2. Scanning electron micrographs of *Betonica bulgarica* Degen et Neič.: A) stem surface; B) abaxial leaf surface; C, D) adaxial leaf surface; E) petiole surface; F) adaxial bract surface; G) abaxial bract surface; H) calyx tube surface; a – non-glandular multicellular uniseriate trichomes, b – peltate glandular trichomes; c – non-glandular stellate multicellular trichomes; d – paracytic stomata; e – anomocytic stomata; f – diacytic stomata.

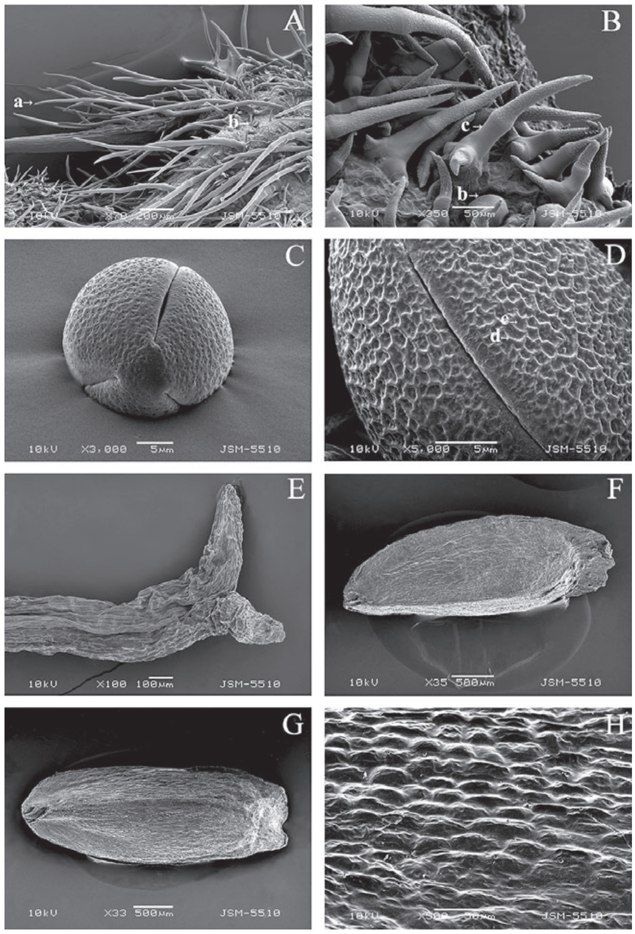

Fig. 3. Scanning electron micrographs of *Betonica bulgarica* Degen et Neič.: A) calyx teeth surface; B) corolla surface; C–D) pollen grains; E) stigmas; F) whole nutlet dorsal face; G) whole nutlet ventral face; H) details of nutlet surface; a – non-glandular multicellular uniseriate trichomes, b – peltate glandular trichomes; c – non-glandular stellate multicellular trichomes; d – muri; e – lumina.

2H and 3B). By the calyx teeth non-glandular multicellular hard non-branched trichomes of up to 2 mm and branched ones with three to five rays were found (Fig. 3A).

Three types of stomata were established in the survey – anomocytic, diacytic and paracytic, the anomocytic being observed most frequently (Tab. 2 and Figs. 2B–D, F–G).

Among the four studied populations no significant variability in pollen morphology was established. The pollens of *B. bulgarica* were monad, medium sized, and 3-zonocolpate (Figs. 3C–D). Polar axis (P) was between 30.2 and 38.5 µm, equatorial axis (E) 20.4–24.2 µm and P/E rate 1.3–1.6. The coloration was reticulate. The width of muri was 0.3–0.6 µm (Fig. 3D). The luminas were slightly elongated with length ranging within 0.8–1.6 µm and width – 0.4–1.2 µm.

The nutlets of *B. bulgarica* were dark brown, triangular, elongated, the outside almost flat, the edges were with narrow wings, which at the top edge went into irregularly toothed membranous appendage (Tab. 2 and Figs. 3F–H). The three sides of the nutlets had even surfaces formed by oblong cells variable in size and usually with no trichomes (Fig. 3G); peltate trichomes were only found on the membranous appendage of single nutlets (Fig. 3F).

Discussion

Intrapopulation variability was dominant in the phenotypic variability of *B. bulgarica* (On-line Suppl. Tabs. 1 and 2). Our studies showed that a possible reason for this was the age structure of the population. Due to the perennial type of the species and the possibilities for seed and vegetative (through roots) reproduction within each population, well-developed perennials were observed. There were also annuals that propagated through seeds and during the first year formed only a leaf patera, as well as biennials that propagated through seeds, which did not always reach flowering and fruiting. In addition, there were vegetatively propagated plants in which the basal leaves were often reduced. Morphometric analyses found that the size of vegetative organs in each of these groups of plants varied significantly within the range of each of the four populations studied. A certain intrapopulation variability was also registered for the size of generative organs in each of the age groups. This determined the dominant role of intrapopulation within total variability along the entire complex of morphological traits.

The height of stem in the different groups in each population varied to a great extent, due to the greatest registered trait variance values (On-line Suppl. Tab. 1). The competitive relations which arise among the specimens of *B. bulgarica* and those of other species in the community, influenced the high variability of that trait. In the populations of Upper Lift Station, Microyazovir and Slancheva Polyana, the uneven distribution of moisture due to the ground slope had an additional effect. It was visible that the specimens located on the steepest parts of the population had shorter stems than those located on even ground.

Higher variability was registered for the traits petiole length of basal and stem leaves (On-line Suppl. Tab. 1). A typical property of the species is that basal leaves always have petioles longer than the laminae, stem leaves have petioles shorter than the laminae and the upper pair are sessile (Koeva 1989). The data from morphometric measurements showed that in some of the specimens the petioles of basal leaves were up to 7 times longer than the lamina and for a period of 20 days their dimensions increased manifold. Since the vegetative development of *B. bulgarica* lasts from the end of March to the first half of April, when the majority of the dominant species are well developed, a rapid extension of leaf petioles results from the competitive relations and provides the leaf laminae with appropriate access to light. Dominant for this trait in the common variability was the interpopulation one (On-line Suppl. Tab. 1) due to the varied composition of communities in the four populations and the different competitive relationships. The greatest amplitude of variability was registered for the population at the Ablanovo area, where the altitude was the lowest and *B. bulgarica* dominated in the grass community (Tab. 1). In specimens that had competitive relations with dominant species of that population, the length of leaf petioles increased up to 2–3 times during a period of 20 days. At the same time in plants that were neighbours to other specimens of *B. bulgarica*, also at the beginning of their vegetative development, the length of leaf petioles and the growth rate were far smaller. The registered high amplitude of variability led to the higher average values of the petiole length of basal and stem leaves for this population. The conducted two-year study showed that in all populations that were in competitive relations with cereals or other plant specimens of *B. bulgarica,* leaf petioles of basal and stem leaves had a greater size. Regardless of the registered variability, the variation in the size of leaf petioles of the studied specimens in the four populations was within the species variability as stem leaves always have shorter petioles than the basal ones and the upper pair of stem leaves are sessile.

Variability was also found for the dimensions of leaf laminae within each population. Plants developing from seeds during the first year had smaller, rounded, elliptic to lanceolate laminae with a blunt tip, scalloped edge and concave base (Figs. 4A–B). Biennial plants produced from seeds (Figs. 4C–D), as well as the other perennial specimens, which reproduced from seeds or vegetatively (Figs. 4E–F), had lanceolate lamina, obtuse apex, asymmetric or concave bases and their dimensions varied. The leaf laminae of biennial plants developed from seeds were always smaller than those of the others (Fig. 4). Regardless of the registered variability in the dimensions of leaf laminae, the ratio between their length and width was one of the traits that had lower variability. With annual specimens that developed from seeds the ratio between length and width of leaf laminae was 1–1.5, and in the other age groups – from 1.5 to 4.5. Regardless of the differences in their size and shape, leaf laminae are always covered with trichomes. The laminae of annual specimens developed from seeds had the thickest trichome cover of all the populations. In specimens from the other age groups no regularities were found in the degree of trichome covering. Variability of basal leaves was established for another species from the genus – *B. officinalis* (Dušek et al. 2010).

Fig. 4. Variation of leaf lamina of *Betonica bulgarica* Degen et Neič.: A–B) annual plants developing from seeds; C–D) biennial plants developing from seeds; E–F) perennial plants.

The dimensions of floral parts (Fig. 5 and On-line Suppl. Tab. 2) in each of the four populations varied slightly and corresponded to those mentioned for the species by Koeva (1989) and Genova (2011). Certain differences were found in the colour of anthers. According to data by Koeva (1989) the stamens of the species have yellow anthers. Our observations showed that the anthers were yellow only immediately after opening of the flower and after that they were purple.

The greatest similarity out of the entire set of quantitative traits (Fig. 1) was registered between the populations at Slancheva Polyana and Microyazovir. In these two populations, the values of the greater part of the studied quantitative traits were very close (see On-line Suppl. Tabs. 1 and 2). Most probably the similarity found was influenced by the similarity in soil type and soil reaction that had contributed to the formation of similar ecological conditions within their boundaries. The soil type in both areas is Eutric Cambisol. The soil is characterized by sandy clay loam soil texture, weakly structured with granular structure and acidic soil reaction (Grozeva et al. 2014).

A certain similarity of the populations from Microyazovir and Slancheva Polyana was found with the population from Upper Lift Station, which has an intermediate altitude compared to the other two populations, significantly smaller area and a different soil type (Fig. 1 and Tab. 1). Domi-

Fig. 5. *Betonica bulgarica* Degen et Neič. – branch with upper leaves and inflorescence (photo M. Srebreva).

nant within the community in all three populations were cereal species, and moisture availability of the soil was lower during the greater part of vegetation.

The greatest difference compared to the populations of Microyazovir, Upper Lift Station and Slancheva Polyana was observed for the Ablanovo population (Fig. 1), where *B. bulgarica* was the dominant species. The area of the population was considerably bigger and the altitude significantly lower (Tab. 1). The soil type also as differed and throughout the entire vegetation soil moisture was moderate. According to our previous investigation (Grozeva et al. 2014), the soil type in that area is Chromic Luvisol (Anonymous 2006). The studied soil is characterized as moderately structured, with crumb structure in the upper surface horizon and a slightly acidic soil reaction with values of pH up to 6.6. The differences in ecological conditions most probably led to the appearance of the registered morphological differences in the species of that population from those of the other three populations. Dušek et al (2010) also evaluated the morphological characteristics between plant populations of different origin from another species of genus *Betonica* – *B. officinalis*.

The most conservative traits in all populations were those characterizing trichomes, stomata, pollen and nutlet morphology (Figs. 2 and 3).

The taxonomic value of the indumentum and its importance in systematic and phylogenetic relationships was well known in Lamiaceae (Abu-Assab and Cantino 1994, Navarro and El Oualidi 2000, Dinç and Őztűrk 2007, Giuliani et al. 2008). Trichomes are among the most useful taxonomic characters for distinguishing the species of genus *Betonica* and genus *Stachys*. The epidermal surfaces of all examined plants of *B. bulgarica* had both non-glandular and glandular trichomes. The peltate and capillate glandular trichomes observed on stem, leaves, bract, calyx and corolla were registered by Giuliani and Maleci Bini (2012) in other species of genus *Stachys* and genus *Betonica*. The peltate trichomes established for *B. bulgarica* (Figs. 2A, C, H, Figs. 3A–B) had the morphological characteristics of the trichomes specified by Giuliani and Maleci Bini (2012) as Type A, whereas the capillate trichomes observed in fresh plants were specified as Type B. According to Giuliani and Maleci Bini (2012) Type A peltate trichomes consist of one basal epidermal cell, one neck cell and a multicellular head with a large subcuticular space where the secretion is accumulated, whereas Type B capillate trichomes consist of one basal epidermal cell, one stalk cell and a head of two to four cells with a thin subcuticular space where secretion is accumulated. Type A trichomes are mainly essential oil producers, whereas Type B have an exclusive or prevalent polysaccharide content (Giuliani et al. 2008). The non-glandular multicellular, simple, uniseriate trichomes observed on stem, leaf petioles, leaves, bracts and calyx tube of *B. bulgarica* were also found by Naidu and Shan (1981) on *B. officinalis*. The non-glandular stellate multicellular trichomes with two to three rays found on calyx and corolla tube on *B. bulgarica* (Fig. 2H, Fig. 3B) were similar to the trichomes on calyx and corolla surface of *B. officinalis* and *B. scardica* as pointed out by Giuliani and Maleci Bini (2012), where, the rays were two to eight. The non-glandular multicellular hard non-branched trichomes up to 2 mm long and branched with three to five rays typical of the calyx teeth of *B. bulgarica* (Fig. 3A) were not mentioned by Koeva (1989) for the calyx teeth of the morphologically closest Bulgarian species – *B. officinalis.* Salmaki et al. (2009) observed numerous types of trichomes of Iranian *Stachys* taxa (incl. *Betonica*) that showed considerable variability among different species, but were constant among different populations of one species, and therefore afford valuable characters in the delimitation of sections and species.

Stomata were described on the leaves of some Lamiaceae by different researchers (El-Gazza and Watson 1970, Inambad and Bhatt 1972, Naidu and Shan 1981). From their study of this family El-Gazza and Watson (1970) reported that the stomata were predominantly diacytic, predominantly anomocytic, predominantly anisocytic and a mixture of anomocytics and anisocitycs. Inambad and Bhatt (1972) found that in 33 Lamiaceae species diacytic was the most frequent type of stomata. Naidu and Shan (1981) pointed out that out of 34 Lamiaceae species the diacytic was the most frequent type, and the anomocytic was the next most abundant type. Our studies confirmed the data known from literature since in all studied plants from the four populations 3 types of stomata have been found – anomocytic, diacytic and paracytic, anomocytic being the most frequent (Figs. 2B–D, F–G). Stomata were evenly distributed throughout the epidermis of abaxial surface without any definite pattern of orientation. According to Naidu and Shan (1981) the anomocytic is the most frequent type in *Stachys officinalis* (syn. *Betonica officinalis*), *S. palustris* and *S. recta.*

Investigations of pollen morphology in Lamiaceae were essential from the point of view of improving the classification within this family (Abu-Assab and Cantino 1994). According to Dinç and Őztűrk (2007) the pollen morphology of the genus *Stachys* (incl. *Betonica*) is not well known. Palynological studies on the Bulgarian *Betonica* taxa have not been conducted so far. Pollen morphology of *B. bulgarica* (Figs. 3C–D) exhibited the features specified by Moore et al. (1991) as *Stachys sylvatica* type. Typical for that type are: trizonocolpate pollens with reticulate ornamentation and lumina more or less uniform in size. Moore et al. (1991) included in the group with *Stachys sylvatica* type pollen eight of the species studied by them, *B. officinalis* among them.

The systematic importance of nutlet morphology in genus *Stachys* and genus *Betonica* was underlined by Demissew and Harley (1992), Martin Mosquero et al. (2000), etc. In a comparison of the nutlet characteristics of *B. bulgarica* with those published by various researchers (Demissew and Harley 1992, Martin Mosquero et al. 2000, Dinç and Doĝan 2006, Salmaki et al. 2008, Satil et al. 2012) for other species in the genus, the greatest similarity according to nutlet shape was established with *B. officinalis*. Regardless of the similarity, nutlets of *B. bulgarica* differed from those of *B. officinalis* by their size (On-line Suppl. Tab. 2) as well as by lack of differences in the reticulate pattern among the three sides of the nutlet surface and the lack of non-glandular trichomes on it (Figs. 3F–H).

In conclusion, the results from the present study demonstrated that the registered intrapopulation variability of the four studied populations of the Bulgarian endemic *B. bulgarica* was based mainly on their age structure, while the established interpopulation variability derived from differences in altitude, soil type and differences in environmental conditions and soil properties. The most taxonomic significance for distinguishing *B. bulgarica* from the other species in the genus, including from the species that is morphologically most similar to it – *B. officinalis* – was found in the indumentum and morphology of generative organs, especially calyx segments and nutlets.

Acknowledgement

This work was financially supported under contract No OPOS 2-36 / 24.07.2013 to project No 5103020-15-658 for Sinite Kamani Natural Park, funded under contract No 5103020-C-002 OPERATIONAL PROGRAMME "ENVIRONMENT 2007- 2013".

References

Abu-Assab, M. S., Cantino, P. D., 1994: Systematic implications of pollen morphology in subfamilies Lamioideae and Pogostemonoideae (Labiatae). Annals of the Missouri Botanical Garden 81, 653–686.

Anchev, M., 2011: General characteristics of Bulgarian flora and floristic regions in Bulgaria. In: Peev, D. (ed.), Red data book of the Republik of Bulgaria. Retrieved from http://e-ecodb.bas.bg/rdb/bg/vol1/4flora.html.

Anonymous, 2002: Biological diversity act. Darzhaven Vestnik no. 77 from 9 August 2002, 9–42. Amended in Darzhaven Vestnik no. 94 from 16 November 2007 (Bg).

Anonymous, 2006: World reference base for soil resources. A framework for international classification, correlation and communication. Food and agriculture organization of the United Nations. Rome, Italy.

Bankova, V., Koeva-Todorovska, Y., Stambolijska, T., Ignatova-Grotseva, M., D., Todorova, D., Popov, S., 1999: Polyphenols in *Stachys* and *Betonica* species (Lamiaceae). Verlag der Zeitschrift für Naturforschung, Tübingen 876–880.

Demissew, S., Harley, R. M., 1992: Trichome, seed surface and pollen characters in *Stachys* (Lamioideae: Labiatae) in Tropical Africa. In: Harley, R. M., Reynolds, T. (eds.), Advances in Labiatae Science, 149–166. Royal Botanic Gardens Kew.

Dinç, M., Őztűrk, M., 2007: Comparative morphological, anatomical and palynologycal studies on the genus *Stachys* L. sect. *Ambleia* Bentham (Lamiaceae) species in Turkey. Turkish Journal of Botany 32, 113–121.

Dinç, M., Doĝan, H. H., 2006: *Stachys yildirimllii* (Lamiaceae), a new species from South Anatolia, Turkey. Annales Botanici Fennici 43, 143–147.

Dušek, K., Duškovă, E., Smĕkalovă, K., 2010: *Betonica officinalis* L. in Czech Republic. I. Variability of morphological characteristics. Herba Polonica 56, 7–20.

El-Gazza, A., Watson, L., 1970: A taxonomic study of Labiatae and related genera. New Phytologist 69, 451–486.

Erdtman, G., 1952: Pollen Morphology and Plant Taxonomy. Angiosperms. Almqvist and Wiksell, Stockholm.

Genova, E., 2011: *Betonica bulgarica* Deg. et Neič. In: Peev, D. (ed.), Red data book of the Republic of Bulgaria, vol. 1. Plants and fungi. Retrieved from http://e-ecodb.bas.bg/rdb/en/vol1/Betbulga.html

Giuliani, C., Maleci Bini, L. 2012: Glandular trichomes as further differential characters between *Stachys* subgenus *Betonica* (L.) Bhattacharjee and *Stachys* subgenus *Stachys*. Plant Biosystems 146, 1–8.

Giuliani, C., Pellegrino, R., Tirillini, B., Maleci Bini, L., 2008: Micromorphological and chemical characterisation of *Stachys recta* L. subsp. *serpentini* (Fiori) Arrigoni in comparison to *Stachys recta* L. subsp. *recta* (Lamiaceae). Flora 203, 376–385.

Grozeva, N., Georgieva, M., Vulkova, M., 2004: Flowering plants and ferns. In: Stoeva, M. (ed.), Biological diversity of Sinite Kamani Nature Park. Kontrast – Bogomilovo, Stara Zagora. 9–112.

Grozeva, N., Todorova M., Gerdzhikova M., Panayotova G., Getova N., Dohchev, D., 2014: New data for Bulgarian endemic *Betonica bulgarica* Deg. et Neič. of Sinite Kamani Natural Park Sliven. Journal of BioScience and Biotechnology 2014, SE/ONLINE, 205–210.

Inambad, J. A., Bhatt, D. C., 1972: Structure and development of stomata in some Labiatae. Annals of Botany 36, 335–344.

Koeva, Y., 1989: Genus *Betonica*. In: Jordanov, D. (ed.), Flora Reipublicae Bulgaricae, vol 9, Editio Acad. "Prof. Marin Drinov", Serdicae, 412–416. (in Bulgarian)

Martin Mosquero, M. A., Juan, R., Pastor, J., 2000: Morphological and anatomical studies on nutlets of *Stachys* (Lamiaceae) from south-west Spain. Thaiszia Journal of Botany 10, 27–38.

Moore, P. D., Webb, J. A., Collinson, M. E., 1991: Pollen Analysis. 2nd edition, 118–122. Blackwell Scientific Publication, Oxford.

Naidu, A. C., Shan, G. L., 1981: Observation on the cotyledonary stomata and trichomes and their ontogeny in some genera of Lamiaceae. Phyton 21, 137–152.

Navarro, T., El Oualidi, J., 2000: Trichome morphology in *Teucrium* L. (Labiatae), A taxonomic review. Anales del Jardín Botánico de Madrid 57, 277–297.

Neichev, I., 1906: Some new for Bulgarian flora plants. Godischnik Sofiiski Universitet 11, 138–144.

Petrova, A., Vladimirov, V., Dimitrova, D., Ivanova, D., 2005: Contemporary state of biodiversity of fern and seed plants in Bulgaria. In: Petrova, A. (ed.), Contemporary state of biodiversity in Bulgaria, 75–104. Drakon, Sofia.

Petrova, A. (ed.), 2006: Atlas of Bulgarian Endemic Plants. Gealibris, Sofia.

Punt, W., Blackmore, S., Nilsson, S., Le Thomas, A., 1994: Glossary of pollen and spore terminology. LPP Foundation. Utrecht.

Salmaki, Y., Zarre, S., Jamzad, Z., 2008: Nutlet micromorphology and its systematic implication in *Stachys* L. (Lamiaceae) in Iran. Feddes Repertorium 119, 607–621.

Salmaki, Y., Zarre, S., Jamzad, Z., Braűchler, C., 2009: Trichome micromorphology of Iranian *Stachys* (Lamiaceae) with emphasis on its systematic implication. Flora 204, 371–381.

Satil, F., Kaya, A., Akçiçek, E., Durmenci, T., 2012: Nutlet micromorphology of Turkish *Stachys* sect. Eriostomum (Lamiaceae) and its systematic implications. Nordic Journal of Botany 30, 352–364.

Velchev, V., (ed.) 1992: Atlas of Endemic Plants in Bulgaria, Bulgarian Academy of Sciences, Sofia.

Yankov, P., (ed.) 2001: National Park Central Balkan. Management plan 2001–2010. Retrieved from http://visitcentralbalkan.net/assets/userfiles/file/Administration/355.pdf.

World reference base for soil resources (WRBSR), 2006: A framework for international classification, correlation and communication. Food and agriculture organization of the United Nations – Rome, Italy.

On–line Suppl. Tab. 1. Mean (upper numbers in each row), variance value (lower numbers in each row, %) and percentage of intrapopulation variation (SSv, %) in the overall morphological variation of *Betonica bulgarica* Degen et Neič. populations for each of the 24 observed vegetative traits; a,b- differences among parameters in the points mentioned with equal letters are not statistically significant.

	Ablanovo area	Slancheva polyana	Upper lift station	Microyazovir	SSv, %
1. Height of stem, cm	73.93 224.24	50.18 158.79	60.84 88.32	46.39 196.51	58.43
2. Number of basal leaves	5.66 2.09	6.22 3.34	6.47 3.59	6.02 5.17	97.46
3. Basal leaves length, cm	11.12 9.70	8.33 10.23	7.40 6.16	8.01 7.16	77.83
4. Basal leaves width, cm	5.75 1.91	4.40 2.10	4.14 1.87	4.00 2.14	77.58
5. Basal leaves length/width ratio	1.95ab 0.16	1.90a 0.15	1.86b 0.17	2.09 0.27	96.60
6. Basal leaves petiole length, cm	26.30 46.83	13.11 21.88	12.29 21.38	11.20 32.53	41.50
7. Stem leaves length, cm	6.03 5.73	5.10 4.95	5.28 4.14	4.79 4.12	95.79
8. Stem leaves width, cm	3.18 2.15	2.91 1.64	2.92 1.45	2.59 1.26	97.46
9. Stem leaves length/width	2.04 0.37	1.87 0.39	1.90 0.25	1.957 0.3	98.68
10. Stem leaf petiole length cm	8.14 31.31	5.77 14.07	6.66 19.90	5.29 14.15	94.40
11. Raceme length, cm	12.81 31.14	7.82 13.98	10.45 71.91	6.08 7.58	82.07
12. First racemes leaves length, cm	2.64 1.22	2.41 1.05	2.88 1.37	2.19 0.85	95.16
13. First racemes leaves width, cm	1.01 0.27	0.89 0.18	1.14 0.27	0.83 0.22	94.86
14. First racemes leaves length/width ratio	2.91a 1.09	2.99a 3.39	2.68 0.40	2.98a 1.34	99.02
15. Raceme leaves petiole length, cm	0.64 0.13	0.39 0.02	0.48 0.10	0.31 0.02	85.41
16. Branch length, cm	13.03 70.88	5.03 7.20	12.86 20.98	4.94 2.78	69.61
17. Raceme branch length, cm	4.00 7.03	1.80 2.04	2.66 0.20	1.44 0.24	73.91
18. Second raceme leaves length, cm	1.35 0.68	1.34 0.57	2.08 0.55	1.37 0.39	84.86
19. Second raceme leaves width, cm	0.44 0.09	0.53 0.11	2.36 9.29	0.53 0.08	77.85
20. Second raceme leaves length/width ratio	3.19 0.43	2.75a 0.61	2.21 1.37	2.71a 0.26	84.81
21. Second raceme leaves petiole length, cm	0.00 0.00	0.21 0.02	0.33 0.49	0.99 0.01	98.18
22. Third raceme leaves length, cm	1.36 0.47	1.00 0.00	1.41 0.19	1.98 7.35	58.69
23. Third raceme leaves width, cm	0.46 0.05	0.30 0.00	0.46 0.01	0.98 0.02	59.76
24. Third raceme leaves length/width ratio	3.05a 0.77	3.33a 0.00	3.14a 0.51	3.00a 0.00	99.28

On–line Suppl. Tab. 2. Mean (upper numbers in each row), variance value (lower numbers in each row, %) and percentage of intrapopulation variation (SSv, %) in the overall morphological variation of *Betonica bulgarica* Degen et Neič. populations for each of the 11 observed generative traits; a,b- differences among parameters in the points mentioned with equal letters are not statistically significant; L/W – length/width ratio.

	Ablanovo area	Slancheva polyana	Upper lift station	Mikroyazovir	SSv, %
25.Calyx length, cm	7.78a 0.25	7.72a 0.46	7.93a 1.61	7.81a 0.75	99.24
26. Calyx width, cm	2.80a 0.75	3.20b 0.56	2.89ab 0.33	3.15ab 0.55	99.24
27. Calyx L/W	2.72a 0.36	2.47b 0.18	2.74ab 0.43	2.43b 0.21	93.21
28. Corolla length, cm	10.73 2.11	11.64a 1.55	11.88a 2.51	11.83a 1.44	89.42
29. Corolla width base, cm	1.06a 0.03	1.09a 0.04	1.09a 0.05	1.10a 0.04	97.49
30. Corola L/W base	10.27 4.08	10.64a 4.63	10.89a 13.15	10.74a 5.16	88.63
31. Corolla width top, cm	5.45a 2.52	5.02a 2.59	4.06 1.08	5.06a 2.77	89.17
32. Corolla L/W top	1.95a 0.13	2.31a 5.94	2.92a 0.21	2.33a 5.94	96.51
33. Nutlets length, mm	3.93a 0.10	3.71a 0.11	3.82a 0.11	3.85 0.18	76.05
34. Nutlets width, mm	1.75a 0.058	1.61b 0.030	1.69ab 0.029	1.77a 0.044	90.66
35. Nutlets length/width ratio	2.24a 0.206	2.38a 0.154	2.27a 0.176	2.22a 0.289	96.82

On–line Suppl. Tab. 3. Factor scores based on principal component analysis and correlations of all *Betonica bulgarica* Degen et Neič. populations – average of 1600 measurements for 35 traits.

	Factor 1	Factor 2	Factor 3
Ablanovo	1.434345	0.3975	–0.18615
Slancheva polyana	–0.38116	0.16364	1.441506
Upper lift station	–0.17819	–1.43334	–0.40469
Microyazovir	–0.875	0.87221	–0.85067