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Morho-anatomical differentiation of the populations of *Daphne cneorum* L. (Thymelaeaceae) from Serbia

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***Abstract*:**

Jušković, M., Vasiljević, P., Savić, A., Jenačković, D., Stevanović, B.: ***Morho-anatomical differentiation of the populations of Daphne cneorum L. (Thymelaeaceae) from Serbia. Biologica Nyssana, 7 (1), September 2016: 1-9.***

In this study was analyzed inter-population diffrentiation of the species *Daphne cneorum* L. on the basis of morpho-anatomical variability of its three different, geographically distant populations, two from Eastern Serbia and one from Western Serbia. The analyzed populations are situated in wide range of altitudes, as well as different geological substrates. The analysis of variance included 20 quantitative characters related to the leaf and stem anatomy and morphology. The results of morphoanatomical studies have shown the presence of general adaptive characteristics of the xeromorphic type. Multivariate analysis have shown a clear distinction between the carbonate populations of *D. cneorum* from eastern Serbia (Mt. Rtanj and Mt. Suva planina) and the serpentine populations from western Serbia (Mt. Zlatibor).

Key words: *Daphne cneorum*, anatomy, morphology, differentiation, Serbia

***Apstrakt*:**

Jušković, M., Vasiljević, P., Savić, A., Jenačković, D., Stevanović, B.: ***Morfo-anatomska diferencijacija populacija vrste Daphne cneorum L. (Thymelaeaceae) iz Srbije. Biologica Nyssana, 7 (1), Septembar 2016: 1-9.***

U ovom radu analizirana je interpopulaciona diferencijacija vrste *Daphne cneorum* na osnovu varijabilnosti morfoloških i anatomskih karakteristika listova i stabla, tri prostorno udaljene populacije, dve iz istočne i jedne iz zapadne Srbije. Analizirane populacije smeštene su u širokom rasponu nadmorskih visina i pokazuju preferencije prema različitim geološkim podlogama. Statističkom analizom je obuhvaćeno 20 kvantitativnih karaktera anatomije i morfologije lista i stabla. Analizom morfo-anatomskih karakteristika listova i stabla i staništnih uslova ustanovljeno je da *D. cneorum* pripada adaptivnom tipu kserofita. Multivarijantne analize su pokazale da se analizirane populacije na osnovu ispitivanih karaktera klasifikuju u dve grupe. Veća međusobna sličnost je prisutna između jedinki iz populacija koje rastu na karbonatu i na većoj nadmorskoj visini, u odnosu na jedinke populacije sa serpentinske podloge i niže nadmorske visine.

Key words: *Daphne cneorum*, morfologija, anatomija, diferencijacija, Srbija

Introduction

Daphne cneorum L. is one of seven species of the genus *Daphne* L. native to Serbia (Blečić, 1972). It belongs to section *Daphnanthes* C.A. Mayer, subsection *Cneorum*, together with *D. arbuscula, D. juliae, D. petraea, D. striata* (Keissler, 1898). Species *D. cneorum* distributed in West, Central and East Europe, Mediterranean region, South–West Asia (Webb & Ferguson, 1968; Meusel et al., 1978). In Serbia, *D. cneorum* is taxon with disjunct disribution and it is limited to the mountainous areas (Blečić, 1972) (**Fig. 1**).

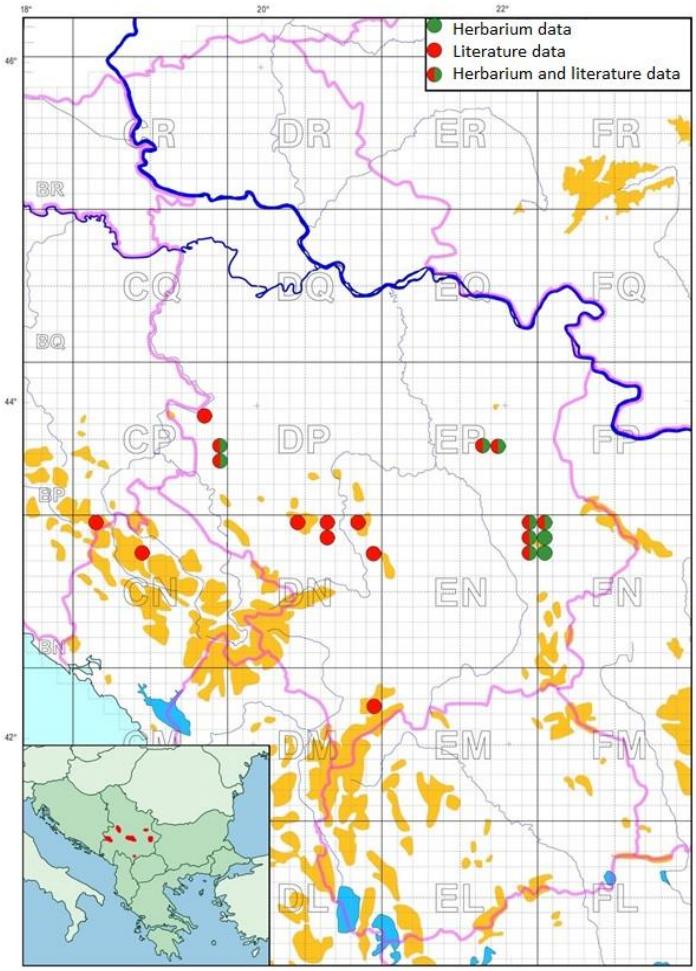


Fig. 1. Distribution of the species *D. cneorum* in Serbia.

The species inhabits mountainous areas, between 700 and 1800 m a.s.l., prefers serpentinite substrate, but it was also recorded on limestone and on acid siliceous soil, usually on stony sites in the zone of *Seslerion rigidae, Seslerio-Festucion, Eu-Vaccinio-Piceenion, Orno-Ericenion serpentinicum, Poion violaceae, Festucion rupicolae, Ramondion nathaliae* alliances (Jušković, 2012). *D. cneorum* is an evergreen fruticose reptant chamaephytes (fo semp Mi Ch frut rept). The species *D. cneroum* has become one of the most popular perennial flowering shrubs, because of their desirable horticultural characteristics (Brickell & White, 1978; Halda, 2001).

Comparative morpho-anatomical studies have involved three different, geographically distant populations, two from Eastern Serbia and one from Western Serbia. The studied populations grew under different climate conditions. The habitats of populations from the area of Western Serbia (Mt. Zlatibor) are influenced by humid temperate-continental climate (type 2.1 Stevanović & Stevanović, 1995; VI 2b Walter & Leith, 1964), with mountain influences, or the transitional variant of temperate-continental and mountain climate of Middle European type (2.1/4.1 Stevanović & Stevanović, 1995; VI 2b/X 1 Walter & Leith, 1964). The populations that inhabit the eastern parts of Serbia (Mt. Suva Planina, Mt. Rtanj) are inflenced by the continental climate (2.2/4.2) This region is dominated by the sub-type of the semi-arid moderately-continental climate, also known as sub-continental climate (type 2.2 sensu Stevanović & Stevanović, 1995; VI 3 sensu Walter & Leith, 1964).

Studies so far on the anatomy of Thymelaeaceae and genus *Daphne* have been limited. Metcalfe & Chalk (1950) give a general description of the anatomical structure of vegetative organs from the family Thymelaeaceae as well as the genus *Daphne*. The species of genus *Daphne* contains significant variation in native habitats, morphology and use (Brickell & White, 1978).

The aim of this study was to determine variability of morpho-anatomical characteristics of the leaves and stems of the plants of species *D. cneorum*, as well as possible trends of differentiations among populations in Serbia.

Material and methods

The species of *Daphne cneorum* were collected from Western (Mt. Zlatibor -serpentinite) and from Eastern Serbia (Mt. Suva Planina, Mt. Rtanj - limestone), during the flowering season in 2008 (**Tab. 1**). The collected plant material was either placed in the herbarium or fixed in 50% ethanol. A voucher specimen was deposited in the Herbarium Moesiacum Niš, Department of Biology and Ecology, Faculty of Sciencesand Mathematics, University of Niš, Serbia (HMN), (**Tab. 1**).

Table 1. Ecological characteristics of the habitats and number of specimens of the analyzed populations *D. cneorum*.

Locality Population	**Biogeography**	**Coordinate**	**Habitat**	**Substratum**	**Altitude (m a.s.l.)**	**Voucher**
Mt. Rtanj, Šiljak Rtanj	Moesian province (CE)	42. 39 N, 19. 39 E	subalpine pastures	limestone	1450-1570	HMN-5515
Mt. Suva Planina, Devojački grob - Trem Suva planina	Moesian province (CE)	43. 11 N, 22. 10 E	rocky grounds	limestone	1647- 1765	HMN-5516
Mt. Zlatibor, Šainovci Zlatibor	Illirian province (CE)	43. 40 N, 19. 41 E	pastures	serpentinite	1000	HMN-5514

Soil analysis

Soil samples were collected from 3 different sites of each of the three population localities. Each analyzed sample contained 300 g of dried, sieved soil. The pH value was determined in a soil suspension with potassium chloride and in one with water (10g : 25cm3), using a potentiometer. The $CaCO_3$ content was determined by the volumetric method, using the Scheibler's calcimeter. The humus content was determined after Turin's method, while that of nitrogen was calculated on the basis of humus values. The available phosphorus was measured by the method of A l l e n (1940) using a spectrophotometer, whereas the available potassium was determined according to the method of Allen on a flame photometer.

Morpho-anatomical analysis

The total of 20 quantitative characteristics subjected to statistical analysis was grouped into two categories: I - morphometric characteristics: II - meristic characteristics. Anatomical sections of leaves and stems was performed on permanent and temporary slides, prepared by the standard histological method for light microscopy (R u z i n , 1999). All measurements were done with on the microscope (Leica DM 250-Leica DFC490, Leica Qwin Standard (Leica Microsystem, Germany). Leaf epidermis, trichomes and stomata were analyzed by a scanning electron microscope (SEM, JEOL 5300).

Statistical analysis

The obtained results of measurements were analyzed in the statistical package SYSTAT 12 (Systat Software Inc. 2007). For each quantitative character was performed basic descriptive statistics: mean, standard deviation, minimum and maximum values. One-way analysis of variance was used in order to calculate statistical significance for analyzed morpho-anatomical characters. Principal component analysis (PCA), canonical discriminant analysis (CDA) and clustering method based on Mahalanobis' distances were used to determine the variability structure and level of importance of the anatomical differentiation of investigated populations.

Results

Soil analysis

D. cneorum inhabits carbonate, silicate, as well as serpentine substrates (B l e č i ć , 1972). Variations in edaphic features, the thickness of the soil, texture, pH values, concentration of minerals, are factors which

Table 2. The results of physico-chemical analyses of the soil samples. The table contains detail informations on pH values, hardness and content of nutrients in soils on the studied habitats.

Locality	**pH**	**$CaCO_3$ (%)**	**Humus (%)**	**N (%)**	**P_2O_5 (mg/g)**	**K_2O (mg/g)**
Mt. Rtanj	6.65	15.81	11.56	0.59	12.4	>40
Mt. Suva planina	6.3	0.72	10.26	0.51	3.6	>40
Mt. Zlatibor	5.99	1.42	7.79	0.39	1.2	20.5

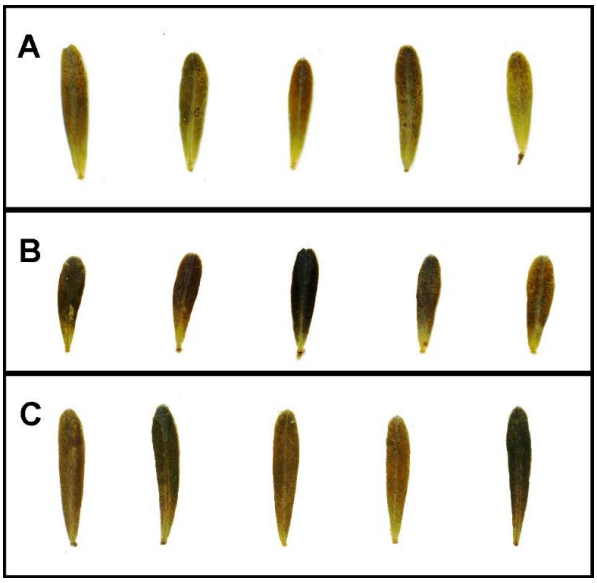


Fig. 2. The leaf shapes of *D. cneorum*: (**A**) Mt. Rtanj, (**B**) Mt. Suva planina, (**C**) Mt. Zlatibor.

influence the distribution of plants (Stevanović & Janković, 2001). The results of soil analysis of the three different sites inhabited by *D. cneorum* are presented in **Tab. 2**. The pH of soil varied from slightly acidic to neutral. Soil is with low content of carbonates on Mts. Zlatibor and Suva Planina, and with high content on Mt. Rtanj. The greatest values of organic matter were recorded in the subalpine pastures soil in the Mt. Rtanj. Chemical analysis shows that there is a sufficient amount of nitrogen. The amount of phosphorus in the soils from the studied localities varied from low to sufficient. Concentration of potassium is sufficient.

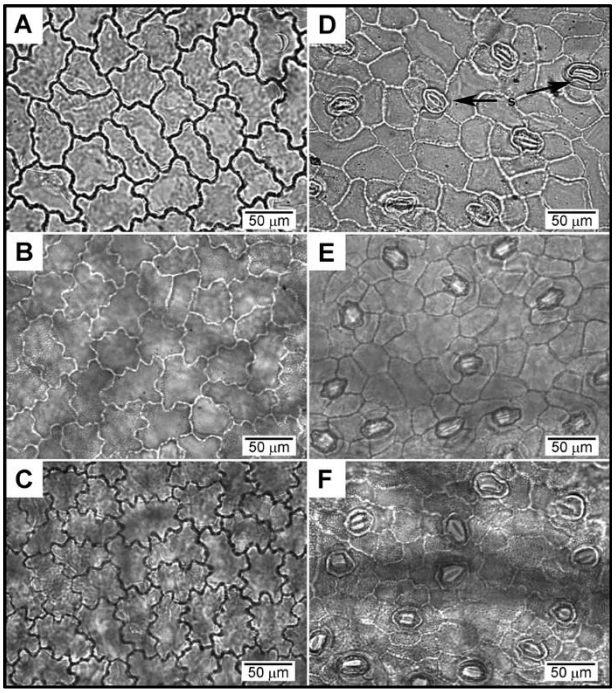


Fig. 3. Epidermal impressions of *D. cneorum* leaves: (**A**, **D**) Mt. Rtanj, (**B**, **E**) Mt. Suva Planina, (**C**, **F**) Mt. Zlatibor: **A-C** adaxial surface, with various shapes of sinuous walls epidermal cells; **D-F** abaxial epidermal cells with straight or slightly sinuous anticlinal walls and anomocytic stomata (**s**).

Leaf shape and anatomy

The leaves of all the analyzed plants species *D. cneorum* are evergreen, simple, narrow, oblanceolate, dark green, with a margin entire and densely arranged towards the end of the twigs. They are with a tapering base and broadly wedge-shaped apex, ending in a minute bristle-like tip. Indumentum is absent from leaves (**Fig. 2**).

The leaf surface area of the analyzed plants from all localities varied, ranging between 28 mm^2 and 67 mm^2, whereas the leaf length was 12 and 22 mm, and the leaf width ranged between 2,5 mm and 4,6 mm (**Tab. 3**). Such microphyllous leaves reduce transpiration rate and represent an important adaptive response of the plants to the conditions of environmental water deficit (Stevanović & Janković, 2001). The highest leaf surface values were from Mt. Zlatibor, while the lowest were in plants from Mt. Suva Planina (**Fig. 2**.; **Tab. 3**).

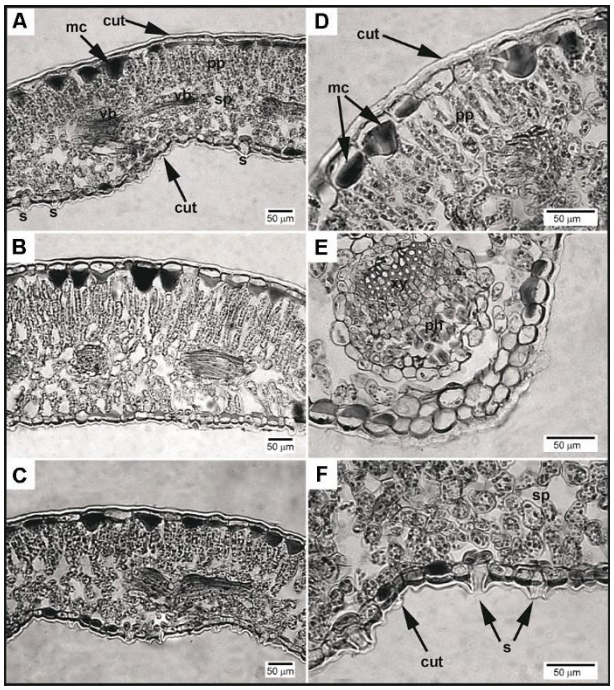


Fig. 4. Cross section of the leaves of plants**. (A)** Mt. Rtanj**, (B)** Mt. Suva Planina, **(C)** Mt. Zlatibor, **(D)** details of the transverse section of the upper leaf epidermis, **(E)** transverse section through the midrib, (**F**) details of the transverse section of the lower leaf epidermis. **cut** cuticule, **mc** mucilaginous cells, **pp** palisade parenchyma, **vb** vascular bundles, **sp** spongy parenchyma, **s** stomata, **xy** xylem, **ph** phloem.

Table 3. Results of One-way Analysis of Variance for comparing means of morphometric and meristic characteristics in three populations of *D. cneorum*. (* p<0.05). Principal component analysis (PCA) of measured parameters of the analyzed populations of *D. cneorum*. The first three principal components accounted for 63.28% of the variance.

Population	Mt. Rtanj	Mt. Suva Planina	Mt. Zlatibor	ANOVA	PCA 1	PCA 2	PCA 3
Character	Mean ± SD	Mean ± SD	Mean ± SD	p-value			
Leaf thickness (µm)	**324.81±45.33**	**316.24±27.82**	**319.69±32.43**	0.65	**0.50**	**0.75**	-0.12
Height of adaxial epidermal cells (µm)	**35.27±4.66**	**34.98±5.49**	**33.70±5.11**	0.45	0.44	**0.74**	-0.14
Thickness of palisade tissue (µm)	**132.75±25.19**	**126.18±21.28**	**122.33±19.23**	0.19	0.39	**0.69**	0.03
Thickness of spongy tissue (µm)	**121.06±19.17**	**109.81±17.92**	**114.43±16.46**	0.06	-0.39	0.22	0.12
Height of abaxial epidermal cells (µm)	**23.65±2.66**	**22.34±2.92**	**21.44±2.30**	0.01*	-0.44	0.08	0.38
Surface area of adaxial epidermal cells (µm^2)	**2105.49±295.47**	**2003.01±234.59**	**2375.47±345.88**	0.00*	0.37	0.16	-0.02
Surface area of abaxial epidermal cells (µm^2)	**932.23±166.27**	**843.79±126.34**	**860.07±112.93**	0.03*	0.47	-0.07	0.26
Surface area of abaxial stomata (µm^2)	**1039.11±87.85**	**977.76±102.26**	**1079.10±86.38**	0.00*	0.08	-0.13	0.10
Leaf length (mm)	**15.43±1.96**	**14.53±1.47**	**18.61±1.70**	0.00*	-0.07	0.44	0.37
Distance between point of largest leaf width and leaf top (mm)	**3.71±0.57**	**3.88±0.56**	**3.39±0.59**	0.51	**0.83**	-0.37	0.16
Largest width of leaf (mm)	**3.45±0.46**	**3.51±0.61**	**3.62±0.35**	0.38	**0.81**	-0.35	0.18
Leaf surface area (mm)	**43.67±9.31**	**39.28±7.93**	**51.01±7.92**	0.00*	**0.64**	-0.22	-0.07
Length of leaf nervature mm/mm^2	**9610.52±1162.67**	**9030.30±1135.57**	**9204.97±822.88**	0.10	0.05	-0.20	**-0.54**
Stem diameter (µm)	**1405.49±202.91**	**1420.04±122.69**	**1396.40±130.73**	0.84	0.19	0.24	-0.28
Stem peridermis thickness (µm)	**27.73±2.86**	**24.37±1.83**	**24.56±2.04**	0.00*	-0.04	0.36	0.44
Stem cortex thickness (µm)	**257.16±25.75**	**284.97±34.46**	**264.12±31.12**	0.00*	-0.05	0.06	**-0.71**
Number of palisade layers	**3.43±0.50**	**3.33±0.61**	**3.50±0.68**	0.56	0.22	-0.31	0.41
Number of abaxial stomata	**131.28±18.57**	**163.18±23.41**	**163.12±22.59**	0.00*	0.15	-0.41	-0.13

The anticlinal cell walls of adaxial epidermis of leaves are wavy with shallow or deep amplitudes, while the anticlinal cell walls of abaxial epidermis are straight to slightly undulate (**Fig. 3**). The undulating anticlinal walls represent a mesomorphic, while straight ones a xeromorphic characteristic (Fahn & Cuter, 1992).

The leaves are bifacial or of dorsiventral symmetry (**Fig. 4**). Their thickness in all the populations studied ranged between from 238 µm to 395 µm (**Tab. 3**). The epidermis of the leaf is single-layered, the adaxial epidermis is thicker than the abaxial epidermis and with well developed cuticule (**Fig. 4**).

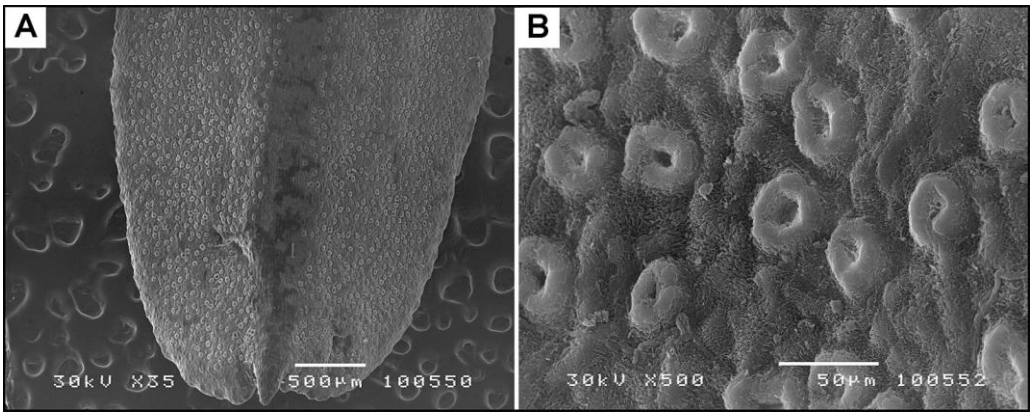


Fig. 5. Scanning electron micrographs showing epidermis of *D. cneorum* leaves, stomata on abaxial leaf surface. (**A**) Mt. Rtanj; (**B**) Mt. Suva Planina (SEM).

The epidermal cell walls are mucilaginous. This type of epidermis is characteristic of the family Thymelaeaceae, as well as the genus *Daphne* (Metcalfe & Chalk, 1950) (**Fig. 4 A-D**). Mucilaginous epidermal cells are usually present mainly in the upper epidermis. Thickened walls of the epidermal cells, well-developed cuticle and mucilaginous cells can prevent rapid water loss and are considered an ecological characteristic for plant species of sunny and dry sites (Bredenkamp, 1999; Bredenkamp & Van Wyk, 2001). Those adaptive strategies limit water loss during periods of drought and such structural adaptations are of major importance to survival in the xerothermic habitat conditions.

Leaves of all studied populations are hypostomatic (**Fig. 4, 5**). The number of stomata in leaves ranges from 95 to 223 per mm^2 (**Tab. 3**). The

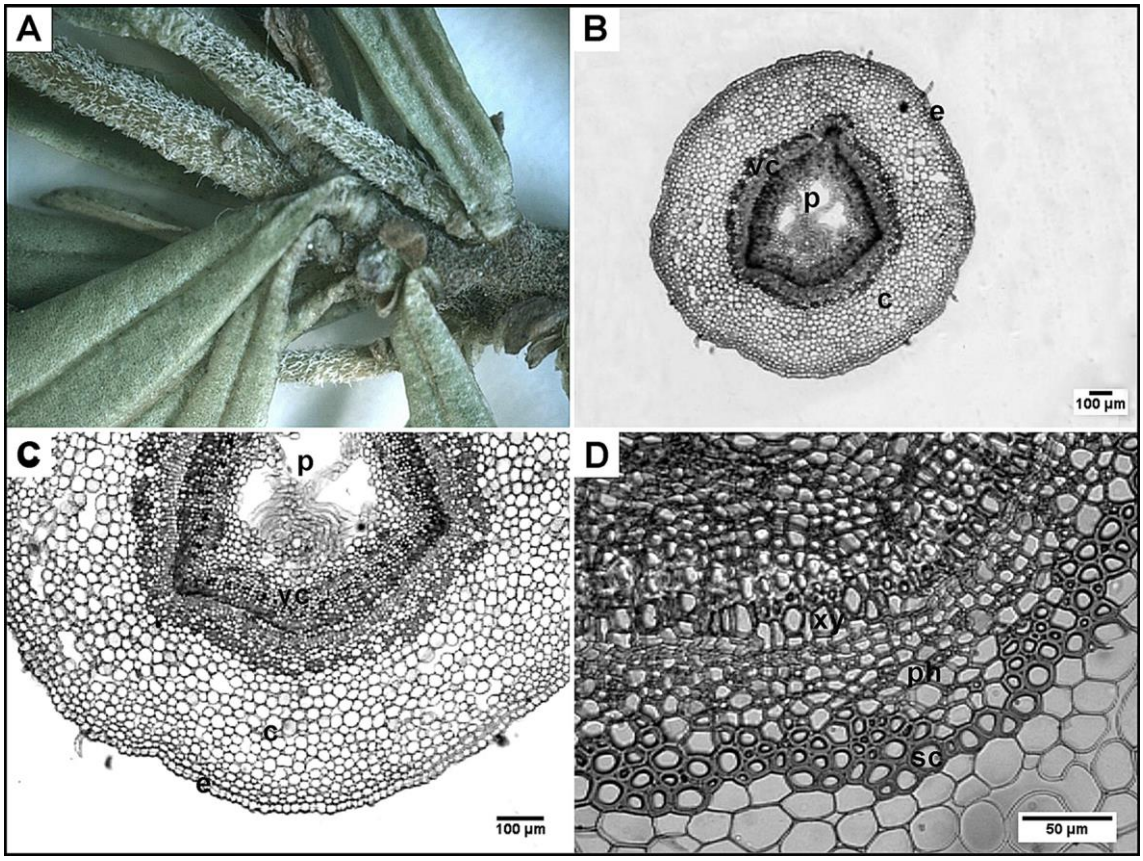


Fig. 6. Cross section of the one-year old stem of *D. cneorum* (in primary state of growth) from Mt. Suva Planina. (**A**) stem with leaves, **(B)** the cross section stem, **(C**, **D)** details of the transverse section of the stem. **e** epidermis, **c** stem cortex, **p** pith, **sc** sclerenchyma cells, **vc** vascular cylinder, **xy** xylem, **ph** phloem.

stomata complexes are anomocytic, i.e., real subsidiary cells are absent (**Fig. 3**). There is high wrinkled cuticule around the stomata (**Fig. 5**). The same type of of stomata were found and described in other species of the genus *Daphne* (Jušković, 2010; Jušković, 2012).

The mesophyll is differentiated into spongy and palisade tissues. The palisade tissue is multilayered, composed of typically elongated, densely arranged cells, with very small intercellular spaces (**Fig. 4**). Incresead toughness of the leaves provides a considerably more efficient water transport and photosythetical light utilization. The cells of spongy parenchyma are usually irregularly shaped. The structure of leaves is involved in the general physiology of plant activity, it clearly explains the morphological adaptation of plants to specific environmental conditions (Stevanović & Janković, 2001). According to Fahn & Cutler (1992) reduced leaf size, increased thickness, deeply sunken stomata, and palisade developed at the expense of spongy mesophyll are common features of plants grown in xeric environments.

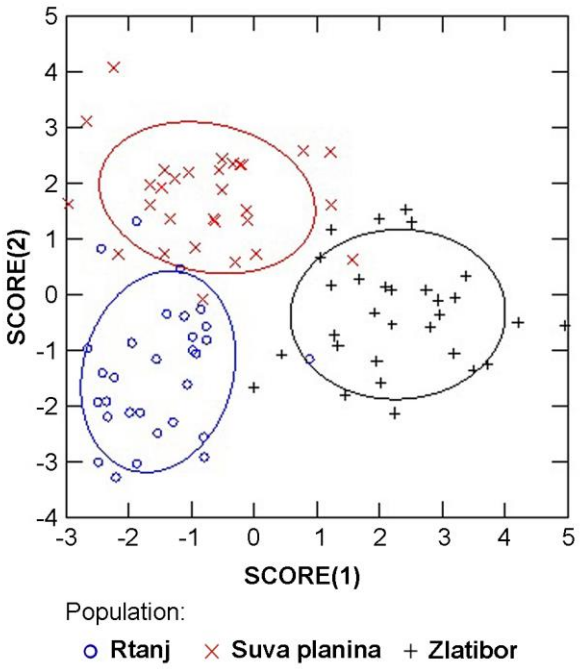


Fig. 7. The result of Canonical discriminant analysis (CDA).

Table 4. Summary of Canonical Discriminant Analysis. Wilks' Lambda is the ratio of within-groups sums of squares to the total sums of squares. The F value for a variable indicates its statistical significance in the discrimination between groups. (* $p<0.05$)

Wilks's Lambda

Lambda	0.09
df	(18. 2. 87)
Approx. F-Ratio	8.70
df	(36. 140)
p-Value	0.00

Variable	F-to-Remove	Tolerance
Leaf thickness	0.81	0.14
Thickness of palisade tissue	0.34	0.22
Thickness of spongy tissue	3.25	0.34
Height of adaxial epidermal cells	0.31	0.80
Height of abaxial epidermal cells	2.38	0.80
Surface area of adaxial epidermal cells	2.15	0.84
Surface area of abaxial epidermal cells	7.46	0.70
Surface area of abaxial stomata	1.17	0.78
Number of palisade layers	0.12	0.85
Number of abaxial stomata	9.08	0.78
Length of leaf nervature	1.41	0.73
Leaf surface area	6.90	0.13
Leaf length	27.38	0.18
Largest width of leaf	2.03	0.35
Distance between point of largest leaf width and leaf top	0.88	0.75
Stem diameter	0.73	0.82
Stem peridermis thickness	7.65	0.89
Stem cortex thickness	2.66	0.81

Stem shape and anatomy

D. cneorum is a low-growing, evergreen shrub, with a great number of long, slender, downy branches and reddish brown bark (**Fig. 6**).

The cross-sections of one year-old stems ranged from rounded to elliptical in shape (**Fig. 6**). Stem diameter of analysis plants ranged between 1396 μm and 1420 μm (**Tab. 3**). The epidermis is uni-layered and covered by a conspicuous cuticle with unicellular trichomes (**Fig. 6A**). Underneath the epidermis, the collenchyma consists of several cell layers (**Fig. 6C**). The cortical parenchyma consists of cells with a circular or polygonal shape and slightly thickened walls. The vascular tissue is well developed and forms a ring around pith. At the outer side of phloem, the sclerenchyma cells also form rings (**Fig. 6D**). The parenchymatous pith has circular or polygonal shaped cells with thin walls. The correlation coefficient of the cortex and the whole stem diameter ranges from od 0,366 (Mt. Rtanj), 0,378 (Mt. Zlatibor) to 0,401 (Mt. Suva planina), which was within the usual values found in xeromorphic stems (Fahn & Cutler, 1992). These characteristics are in agreement with the data obtained from other studied *Daphne species* (Jušković, 2010; Jušković, 2012).

Finally, leaf and stem anatomical features are closely linked to the habitat of species *D. cneorum*, indicating that they represent ecologically important adaptations. On the other hand, phenotypic plasticity among populations of the same plant species is one of the important mechanisms for their adaptation to heterogeneous habitats, and it is one of the major way by which plants are able to cope with environmental variability (Jušković et al., 2010; Lakušić et al., 2010; Jakovljević et al., 2013; Gratani, 2014).

There is statistical significant differences among populations according to some morpho-anatomical features: height of abaxial epidermal cells, surface area of adaxial epidermal cells, surface area of abaxial epidermal cells, surface area of abaxial stomata, leaf length, leaf surface area, stem peridermis thickness, stem cortex thickness and number of abaxial stomata among populations (**Tab. 3, 4**).

The results of PCA analysis showed that the three principal components explain 63.28% of the total variability (**Tab. 3, 4**). The largest contribution to the formation of variability provides the first principal component with 32.78%, followed second with 17.29% and the third with a 13.51% variation. The greatest significance in the formation of variability have characters that are related to leaf shape (leaf surface area, largest width of leaf, distance between point of largest leaf width and leaf top) and leaf anatomy (leaf thickness, height of adaxial epidermal cells, thickness of palisade tissue). Stem cortex thickness is present in the structure of the variability in the third principal component.

The results of canonical discriminant analysis of the population of the species *D. cneorum* show the existence of separation among the studied populations (**Fig. 7**). Population with Mt. Zlatibor is on the positive side of the first axis and is clearly separated from the population with Mt.Rtnja and Mt. Suva Planina. Less discrimination is present in populations from eastern Serbia, which are the negative side of the first axis. The most important characters (**Tab. 4**) in total discrimination among populations are characters related to the leaf shape, leaf epidermis and stem peridermis thickness.

Multivariate analysis (PCA and CDA) and cluster analysis (**Fig. 8**) have shown that studied populations can be classified into two groups. One group includes populations (Mt. Rtanj and Mt. Suva Planina) that grow on carbonate substrate, and at a higher altitude (1500-1700 m a.s.l.), while the second group includes plants from the population (Mt. Zlatibor), growing on serpetinite substrate and the lower altitude (1000 m a.s.l.).

Conclusion

The investigated populations of the species *Daphne cneorum* have shown certain differences in structure. The results of morpho-anatomical studies of the leaves and stems, and conditions on habitats, have shown the presence of general adaptive

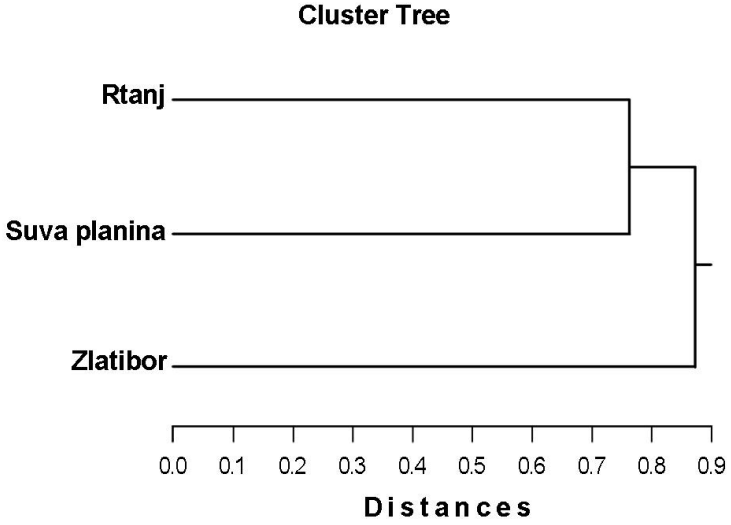


Fig. 8. Mahalnobius distances among three populations of *Daphne cneorum*.

characteristics of the xeromorphic type. The study provides useful information on the morpho-anatomical and ecological characteristics of species *D. cneorum*.

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