Botanica SERBICA 34 (2): 127-132 (2010)

Original Scientific Paper

On the taxonomical identity of *Abies alba* ssp. *borisii-regis* (Mattf.) Koz. et Andr.– morphometry, flavonoids and chorology in Bulgaria

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ABSTRACT: Thirty four samples of *Abies alba* ssp. *borisii-regis* (Mattf.) Koz. et Andr, *Abies alba* Miller. and *Abies cephalonica* Loudon collected from Rila, Pirin, Western Rhodopes, Slavyanka, Black Sea coast and Greece have been subjected to variation and cluster analyses. *Abies alba* ssp. *borisii-regis* is mentioned in Bulgarian Red list under LC category. In the same time this species is a major species to the endemic habitat 9270 Hellenic beech forests with *Abies borisii-regis* distributed in Southern Bulgarian mountains and some other mountains of Balkan peninsula. The obtained results support the idea about intermediate character of *Abies alba* ssp. *borisii-regis* (between *A. alba* and *A. cephalonica*) and corresponding species rank - *Abies borisii-regis*.

Key words: cluster analysis, *Abies alba* ssp. *borisii-regis*, *Abies alba*, *Abies cephalonica*, variation coefficient, flavonoids.

Received 12 October 2009 Revision accepted 18 June 2010

UDK 582.475(497.2) ; 519.237.8

INTRODUCTION

The taxon *Abies alba* ssp. *borisii-regis* (Mattf.) Koz. et Andr. is given as subspecies in Bulgarian flora. Its distribution in Bulgaria is in the mountains in the South part of the country (Slavyanka, Belasitsa, Southern Pirin, Middle and Western Rhodopes, Southwestern Rila) in area of elevation between 900 and 1800 m. (Andreev 1992). The subspecies take part in the threatened habitat of 9270 Hellenic beech forests with *Abies borisii-regis*. It is included in the Red List in Bulgaria under the category of Least Concern (LC). (Petrova & Vladimirov 2009). In Flora Europaea the species rank status was adopted – *A. borisii-regis* Mattf. It is distributed in the Balkan Peninsula from Southern Albania to the Thrace region. The species has many varying forms, but always more or less intermediate between *A. alba* Miller and *A. cephalonica* Loudon. It is most likely of hybrid origin (Chater 1964). Our goal is to verify the statistical isolation and partially the phytochemical isolation of *Abies alba* and *Abies alba* ssp. *borisii-regis*.

MATERIALS AND METHODS

Plant material. Thirty four individual representative samples of *Abies alba* Mill. (16 samples), *A. alba* ssp. *borisii-regis* (Mattf.) Koz. et Andr. (14 samples) and *A. cephalonica* Loudon. (3 samples) have been subjected to Variation and Cluster analyses. The origin of the samples was from 5 floristic regions of Bulgaria: Rila, Pirin, Western Rhodopes, Slavyanka, Black Sea coast (north) and one sample from Greece. (Table 1)

Variation and cluster analysis. Four morphometric features were analyzed as follows: x_1 – diameter of the circular-shaped base of the needle, x_2 – needle length from the base, x_3 – maximum needle width, x_4 – thickness in the middle of the needle. The morphometric features x_1, x_2 and x_3 were measured with accuracy of up to 0.05 mm and the feature x_4 - 0,025. Hundred needles of each sample have been measured. The degree of variation was measured according to the variation coefficient scale (Peev 1985):

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Table 1. Origin and taxonomy of the analyzed samples

Sample (№)	Taxon	Floristic regions	Location	Date of sampling	m above sea level; longitude and latitude
1	*A. alba* ssp. *borisii-regis*	Rila	Hotel 'Alen Mak'	02.04. 2009	1716 m; 42° 12,718' N/23° 23,259' E
2	*A. alba* ssp. *borisii-regis*	Rila	Hotel 'Malyovitsa'	02.04. 2009	1709 m; 42° 12,659' N/ 23° 23,266' E
3	*A. alba* ssp. *borisii-regis*	Rila	Hotel 'Malyovitsa'	02.04. 2009	1709 m; 42° 12,659' N/ 23° 23,266' E
4	*A. alba* ssp. *borisii-regis*	Rila	hut 'Malyovitsa'	02.04. 2009	1906 m; 42° 11,683' N/ 23° 22,732' E
5	*A. alba*	Rila	Mecha polyana	02.04. 2009	1733 m; 42° 12,466' N/ 23° 23,250' E
6	*A. alba* ssp. *borisii-regis*	Western Rhodopes	By Yadenitsa river, at 15 km northern from Golyamo Belovo	06.04. 2009	1129 m; 42,10493° N/ 23,90043° E
7	*A. alba* ssp. *borisii-regis*	Western Rhodopes	By Yadenitsa river, at 15 km northern from Golyamo Belovo	06.04. 2009	1129 m; 42,10493° N/ 23,90043° E
8	*A. alba*	Rila	Hotel 'Alen Mak'	02.04. 2009	1716 m; 42° 12,718' N/ 23° 23,259' E
9	*A. alba*	Rila	Hotel 'Alen Mak'	02.04.2009	1716 m; 42° 12,718' N/ 23° 23,259' E
10	*A. alba*	Western Rhodopes	By Yadenitsa river	06.04. 2009	1129 m; 42,10493° N/ 23,90043° E
11	*A. alba*	Western Rhodopes	By Yadenitsa river	06.04. 2009	1129 m; 42,10493° N/ 23,90043° E
12	*A. cephalonica*	Greece			
13	*A. cephalonica*	Town of Zemen	The tree planted in the Town Hall yard	12.06.2009	596 m; 42° 28,686' N/ 23° 44,831' E
14	*A. alba* ssp. *borisii-regis*	Slavyanka	Hambar dere	1.07.2009	1000 m
15	*A. alba*	Western Rhodopes	Direction to Sarnitsa Town	16.05.2009	1632 m; 41,89093° N/ 23,93705° E
16	*A. alba*	Western Rhodopes	By Yadenitsa river	16.05.2009	1129 m; 42,10493° N/ 23,90043° E
17	*A. alba*	Western Rhodopes	At 10 km from Velingrad Town, to Yundola	16.05.2009	988 m; 42,03136° N/ 23,90343° E
18	*A. alba*	Western Rhodopes	By the road between the Chairite Dam and the Sestrimo village	21.06.2009	1050 m; 42° 11,215' N/23° 52,952' E
19	*A. alba*	Western Rhodopes	Krastava place	16.05.2009	1023 m; 41,93547° N/ 23,92017° E
20	*A. alba*	Western Rhodopes	Sofan dere	16.05.2009	1056 m; 41,93153° N/ 23,92623° E
21	*A. alba*	Western Rhodopes	5 km before Yundola on the road from Belovo	19.06.2009	1270 m; 42° 05,442' N/ 23° 53,302' E
22	*A. alba*	Western Rhodopes	Grashevo Village	16.05.2009	1295 m; 42,08445° N/ 23,88552° E
23	*A. alba*	Pirin	hut 'Gotse Deltchev'	21.06.2009	1479 m; 41° 45,608' N/ 23° 32,814' E
24	*A. alba*	South Pirin	Above Papaz Chayir, near Orelek peak	6.07.2009	1524 m; 41° 33,101' N/ 23° 37,722' E
25	*A. alba*	South Pirin	Popovi livadi	18.07.2009	1432 m; 41,33152° N/ 23,38138° E

Sample (№)	Taxon	Floristic regions	Location	Date of sampling	m above sea level; longitude and latitude
26	*A. cephalonica*	Northern Black Sea coast	Specimen planted in the University Botanic Garden in town of Balchik	24.07.2009	13 m; 43° 24,251' N/ 28° 8,917 E
27	*A. alba* ssp. *borisii-regis*	Rila	On the trekking course between the Rila Monastery and hut 'Iv.Vazov'.	17.07.2009	1544 m; 42,07614°N/ 23,19240° E
28	*A. alba*	Rila	On the trekking course between the Rila Monastery and Hut 'Iv.Vazov'.	17.07.2009	1307 m; 42,07283° N/ 23,19747° E
29	*A. alba* ssp. *borisii-regis*	Rila	9 km off village of Pastra, on the way to Kalin Dam.	1.08.2009	1616 m; 42° 09,489 ,N/ 23° 13,579' E
30	*A. alba* ssp. *borisii-regis*	Rila	9 km off village of Pastra, on the way to Kalin Dam.	1.08.2009	1616 m; 42° 09,489 ,N/ 23° 13,579' E
31	*A. alba* ssp. *borisii-regis*	Rila	3 km off Kirilova polyana, on the way to Ribni lakes.	1.08.2009	1527 m; 42° 09,334 ,N/ 23° 24,582' E
32	*A. alba* ssp. *borisii-regis*	Rila	Kirilova polyana	1.08.2009	1475 m; 42° 09,301 ,N/ 23° 24,007' E
33	*A. alba* ssp. *borisii-regis*	Rila	Kravarsko dere	1.08.2009	1587 m; 42° 16,371 ,N/ 23° 19,994' E
34	*A. alba* ssp. *borisii-regis*	Slavyanka	Above Parilska saddle	6.08.2009	1625 m; 41°24' 37,3"N/23° 38' 54,3"E
35	*A. cephalonica*	Znepole Region	At 3 km Eastern from Tran Town	5.01.2005	800 m; 42.83479° N/22.69361° E

V<7% - very low, 7,1<V<12% - low, 12,1<V<20% - medium, 20,1<V<40% - high, V>40,1% - very high. The obtained data were subject to cluster analysis (Nearest Neighbor Method, Euclidean distance), whereby the clusters were formed by calculating the degree of similarity, based upon the average values.

Flavonoid analysis.

Preparation of plant extracts. Ground plant material (1g) was extracted twice with 30 ml 80% metanol. The extract was then evaporated and transferred to a separating funnel. Water (50ml) was added and extraction consecutively with dichloromethane and ethyl acetate was repeated 2 times with 20 ml each. The ethyl acetate fractions were collected and evaporated to dryness. The residue was dissolved in methanol and the solution was used for analyses of flavonoids.

Total flavonoid content. For analysis on total flavonoid content, the dried extract was diluted in methanol to a final concentration of 1mg/ml. Then 0.5ml of this extract was mixed with 1 ml 2% $AlCl_3$ and the absorbance of this solution was measured at 426nm. Measurement of the solution before addition of Neu reagent serves as a blank (Bahorun *et al.* 2004). Given that quercetin was the main component, flavonoids were expressed in terms of quercetin equivalent. Extracts were analyzed with a Jenway6320D spectrophotometer.

TLC analysis of flavonoid components. The identification of flavonoid aglycones was determined by co-chromatography with reference compounds. Two TLC systems confirmed the identification of the flavonoids. System 1: silica gel, toluene–dioxane–acetic acid 95 : 25 : 4 v/v/v; 2: cellulose plates, acetic acid–water 60–30 v/v.

RESULTS AND DISCUSSION

Variation analysis

Morphometric features. (Table 2) Diameter of the circular-shaped base of the needle (x_1). This feature had a diameter of 0,4 mm to 4,1 mm. The lowest average value was observed in sample 14 (*A. alba* ssp. *borisii-regis*), and the highest one – in samples 12 and 26 (*A. cephalonica*). In samples 6 and 27 (*A. alba* ssp. *borisii-regis*) there was a substantial interruption (20%) in the maximum of the series of standard deviation values, which revealed its greater variability.

Needle length from the base (x_2). In our samples, the length of the needles varied between 8,2 mm and 41,5 mm. The lowest average values were seen in samples 12 and 26 (*A. cephalonica*) and the highest one – in sample 21 (*A. alba*). Regarding this features, there were no interruptions in the series of standard deviation values.

Maximum needle width (x_3). The needles width were between 0,35 mm and 2,8 mm wide. The lowest average

Table 2. Summary table presenting the dimensions and the standard deviation of the morphometric features

Features	Sizes (mm)				Average				Standard deviation			
	minimum		maximum		minimum		maximum		minimum		maximum	
	Value	Sample	Value	Sample	Value	Sample	Value	Sample	Value	Sample	Value	Sample
X_1	0.4	10	4,1	27	0,776	14	1,586	26	0,101	1	0,367	27
X_2	8,2	12	41,5	21	15,398	26	29,665	21	2,158	26	5,486	19
X_3	0,35	27	2,8	8	1,566	20	2,279	31	0,107	1	0,232	27
X_4	0,05	8	1,45	13	0,211	8	0,643	26	0,038	10	0,172	33

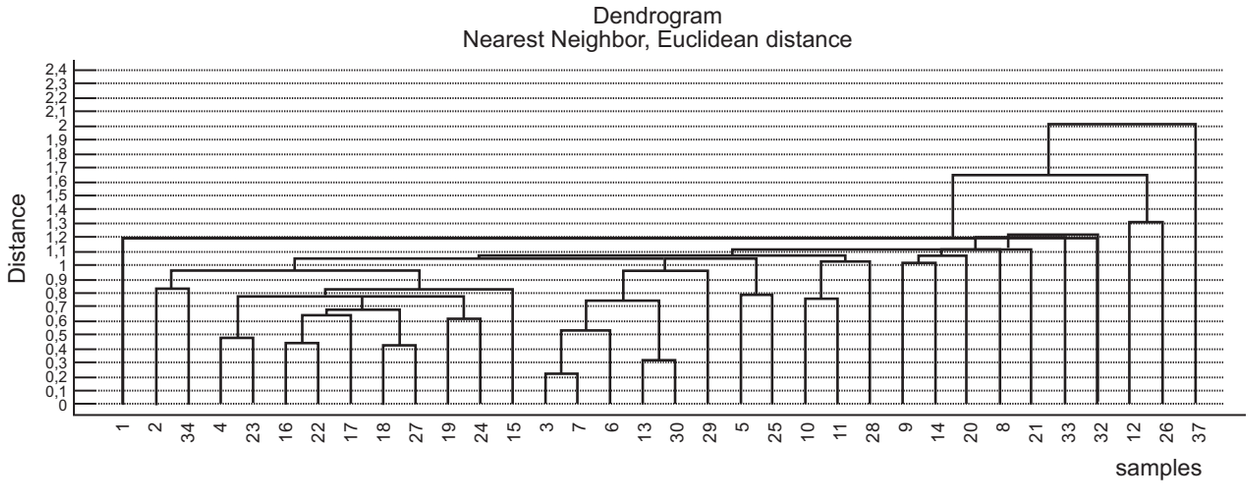

Fig. 1. Groups among all samples of *Abies*

values were seen in samples 9 and 20 (*A. alba*) and the highest ones – in samples 31 and 32 (*A. alba* ssp. *borisii-regis*). There was an interruption in the standard deviation value series in sample 27 (*A. alba* ssp. *borisii-regis*).

Thickness in the middle of the needle (x_4). The needle thickness of the samples analyzed varied between 0,05 mm and 1,45 mm. The lowest average value was observed in sample 8 (*A. alba*) and the highest one – in sample 26 (*A. cephalonica*). There were two interruptions in the maximums of the standard deviation series. The smaller one was in sample 26, and the greater dispersion in maximum was in sample 33 (*A. alba* ssp. *borisii-regis*).

Variation coefficients. In analyzed samples, variation coefficients were in the low (27%), medium (60%) and high (13%) degrees. (Table 3) No coefficients are found within the very low or within the very high variability degree. Low variability is registered for the needle width (x_3); medium variability was observed in the size of the diameter of the circular-shaped base of the needle (x_1) and the needle length (x_2), and there was a relatively high degree of variability in the thickness of the needles (x_4).

Cluster analysis. The cluster analysis reveals a relatively high degree of metric similarity within the limits of 0,22 to 1,22. Despite that, a group was formed of *A. cephalonica* at a significant distance (1,64) from the other samples. There are significant difference between both samples of *A. cephalonica*. There were five main subgroups in the cluster:

The first subgroup was composed of 12 samples with a minimum distance of 0,46 and a maximum distance of 0,96. It included 4 samples of A. alba and 8 samples of *A. alba* ssp. *borisii-regis*. Those were from different mountains and zones of varied elevation.

The second subgroup was at a minimum distance of 0.22 and a maximum distance of 1,08. It was composed of 11 samples, 5 of which belonged to *A. alba* ssp. *borisii-regis*, 5 belonged to *A. alba* and 1 to *A. cephalonica*. Their origins were from different floristic regions. In this group

Table 3. Variation coefficients of *Abies* (CV%)

Features Samples	X_1	X_2	X_3	X_4
1	11,82	14,09	6,23	16,3
2	11,64	17,8	8,52	22,29
3	18,14	18,64	8,46	25,5
4	18,22	16,73	7,61	26,48
5	16,62	20,21	10,29	27,32
6	18,57	17,64	11,2	17,62
7	16,87	14,47	8,48	19,08
8	13,17	16.50	9,29	26.00
9	15,83	15,1	7,29	23.80
10	20,03	17,22	10,3	15,41
11	13,87	17,55	10,78	21,61
12	14,73	16,84	6,76	16,46
13	18,93	18,02	6,77	25,5
14	13,84	19,26	9,22	17,4
15	14,63	16,48	8	14,28
16	16,25	21,79	9,61	17,71
17	15,33	19,25	8,83	16,02
18	15,00	15,78	5,91	13,78
19	12,82	22,08	8,94	13,14
20	14,45	18,88	7,16	28,03
21	14,56	18,09	10,35	13,59
22	13,29	16,27	8,2	16,88
23	14,94	16,84	6,81	19,51
24	13,04	14,8	6,79	15,6
25	11,14	13,06	7,19	21,31
26	13,2	14,02	8,93	18,98
27	35,91	18,76	11,48	20,78
28	12,39	19,72	9,43	15,17
29	11,32	15,64	8,76	12,98
30	19,02	20,72	9,42	15,09
31	15,68	17,65	6,61	21,79
32	12,47	20,69	7,43	22,51
33	16,93	18,36	7,33	30,03
34	17,32	15,4	6,9	18,37

two pairs of individual samples from the same elevation were at a close distance.

The third subgroup was at a minimum distance of 0,02 and a maximum distance of 1,22. It was composed of 5 samples: one of them was of *A. alba* ssp. *borisii-regis*, and the rest – of *A. alba*. The samples were from different regions and elevations.

The fourth subgroup was composed of 3 samples of *A. alba* ssp. *borisii-regis*, which were connected in the cluster of relatively greater distance of 1,20 to 1,24 than the previous three subgroups. Samples were from different regions of the Rila Mountain.

The fifth subgroup had a minimum distance of 1,32 and a maximum distance of 1,64. It was composed of two samples of *A. cephalonica* from Greece and the Black Sea coast and 1 sample of the *A. alba* ssp. *borisii-regis* from Rila (Kirilova polyana). This fact shall be interpreted as additional evidence for the intermediate character of *A. alba* ssp. *borisii-regis* (between *A. alba* and *A. cephalonica*.)

Flavonoid analysis. Twenty samples of *Abies* species were studied for their flavonoid content by TLC analysis and spectrophotometric assay (Table 4). Three flavonoid aglycones quercetin, isorhamnetin and myrecetin were identified by TLC but there are other spots with flavonoid behavior that rest unidentified. The samples of *A. chephalonica* are differed of the samples of *A. alba* on their flavonoid profiles. The extracts of *A. chephalonica* contain isorhamnetin and the accumulation of quercetin and miricetin are higher than these of *A. alba* samples. The samples of *Abies alba ssp. borisii-regis* and *A. alba* have similar flavonoid profiles. The study on the total flavonoid content showed that there no correlation between total flavonoid content and species belonging of the samples.

Table 4. Flavonoids in the samples of Abies species

Legend: * expressed as mg quercetin equivalents / g EtOAc extract**; tr- trace**

Abies species	**No of sample see Table1**	**Flavonoid aglycones**			**Total flavonoids***
		quercetin	**isorhamnetin**	**miricetin**	
*A. cephalon*ica	12	++	++	++	0.667±0.04
A. cephalonica	35	+	+	+	0.546±0.05
A. cephalonica	13	+	tr	+	0.386±0.08
A. cephalonica	26	+	tr	+	0.319±0.10
A. alba ssp. *borisii-regis*	2	tr	-	tr	0.446±0.11
A. alba	5	tr	-	tr	0.442±0.12
A. alba ssp. *borisii-regis*	7	tr	-	tr	0.456±0.09
A. alba	10	tr	-	tr	0.328±0.08
A. alba ssp. *borisii-regis*	6	tr	-	tr	0.361±0.10
A. alba	16	tr	-	tr	0.360±0.08
A. alba ssp. *borisii-regis*	1	tr	-	tr	0.458±0.11
A. alba	8	tr	-	tr	0.277±0.08
A. alba	23	tr	-	tr	0.340±0.08
A. alba ssp. *borisii-regis*	27	tr	-	tr	0.356±0.09
A. alba	28	tr	-	tr	0.337±0.11
A. alba ssp. *borisii-regis*	30	tr	-	tr	0.347±0.08
A. alba ssp. *borisii-regis*	32	tr	-	tr	0.333±0.09
A. alba ssp. *borisii-regis*	33	tr	-	tr	0.291±0.11
A. alba ssp. *borisii-regis*	34	tr	-	tr	0.398±0.11

CONCLUSION

In the measurements carried out, *A. cephalonica* discreetly stands out with a greater diameter of the circular-shaped base of the needle and with the shortest and thickest needles. This difference is also visual by its xeromorphic type.

In both other taxa no significant differences of the features measured were observed.

A higher degree of variability was registered for *Abies alba* ssp. *borisii-regis* in needle thickness (x_4). The other features revealed a low degree of variability. More significant interruptions in the standard deviation values were observed in the maximums of features x_1, x_3 and x_4 for samples belonging to taxon *A. alba* ssp. *borisii-regis*.

The metrical variability observed gives us grounds to regard both ecotypes (*Abies alba* ssp. *borisii-regis* and *Abies alba*) as ecotypes with medium variability. This is related to the similar climate conditions at the habitats.

A relative metrical similarity between taxa in terms of the features analyzed was observed. This similarity was measured by means of the relatively small Euclidean distances from the degree of total similarity.

Based on the analysis of the features studied, there was no morphometrical isolation between the samples of *Abies alba* ssp. *borisii-regis* and *Abies alba*.

Notwithstanding the relative difference between them, both samples of *A. cephalonica* formed a separate group in a common cluster.

The fact that samples of *Abies alba* ssp. *borisii-regis* fall into the same subgroup with *A. cephalonica* can be interpreted as support for the hypothesis about the intermediate character of *Abies alba* ssp. *borisii-regis*.

The extracts of *Abies alba* ssp. *borisii-regis* and *A. alba* have similar flavonoid profiles whether the flavonoid profiles of *A. chephalonica* extracts are differed by presence of isorhamnetin and higher quantity of quercetin and miricetin.

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REZIME

O taksonomskom identitetu *Abies alba* ssp. *borisii-regis* (Mattf.) Koz. et Andr.– morfometrija, flavonoidi i horologija u Bugarskoj

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Proučavana je varijabilnost trideset i četiri uzoraka *Abies alba* ssp. *borisii-regis* (Mattf.) Koz. et Andr, *Abies alba* Miller. i *Abies cephalonica* Loudon sakupljenih sa lokaliteta na Rili, Pirinu, Zapadnim Rodopima, Slavjanki, Crnomorskoj obali i Grčkoj i data je klaster analaiza. *Abies alba* ssp. *borisii-regis* pominje se u Bugarskoj crvenoj listi u kategoriji LC. Istovremeno, vrsta je dominantna na endemičnom staništu "9270 Helenskih bukovih šuma" sa *Abies borisii-regis*, a ovakve sastojine javljaju se i na planinama u južnoj Bugarskoj i drugde na Balkanskom poluostrvu. Dobijeni rezultati podržavaju tezu o inetermedijernom karakteru *Abies alba* ssp. *borisii-regis* (izmedju *A. alba* i *A. cephalonica*) i odgovaraju nivou vrste *Abies borisii-regis*.

Ključne reči: klaster analiza, *Abies alba* ssp. *borisii-regis*, *Abies alba*, *Abies cephalonica*, koeficijent varijacije, flavonoidi