Variability of headspace volatiles in native population of *Abies* x *borisii-regis* from the central Rhodopes

Original Article

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Abstract:

The present study investigates variability of headspace needle volatiles in native population of *Abies* x *boisii-regis* (King Boris fir) from the central Rhodopes (southern Bulgaria) as well as the relationships among this and seventeen previously studied fir populations from the presumed zones of natural hybridization in the Balkan Peninsula. According to multivariate statistical analyses, all four populations identified as *A.* x *borisii-regis* from southern Bulgaria, northern and central Greece included volatile profiles of both supposed parent species (*A. alba* and *A. cephalonica*), where profile frequencies changed clinally along the latitudinal gradient. Considering that one of the indicators of the presence of a hybrid zone is the clinical variation of all or a larger number of characters located in the same area, the observed geographic distribution of volatile entities supports the hypothesis that studied *A.* x *borisii-regis* populations are of secondary origin due to hybridization between *A. alba* and *A. cephalonica*.

Key words:
Abies x *borisii-regis*, Rhodopes, headspace, needle volatiles, chemodiversity

Apstract:

Varijabilnost hedspejs isparljivih jedinjenja u prirodnoj populaciji *Abies* x *borisii-regis* sa centralnih Rodopa

Ovo istraživanje ispituje varijabilnost hedspejs isparljivih jedninjenja četina prirodne populacije *Abies* x *borisii-regis* (jela kralja Borisa) sa centralnih Rodopa (južna Bugarska), kao i odnose između ove i 17 prethodno istraživanih populacija jela iz pretpostavljene zone prirodne hibridizacije na Bakanskom poluostrvu. Na osnovu multivarijantnih statističkih analiza, sve četiri populacije identifikovane kao *A.* x *borisii-regis* iz južne Bugarske, severne i centralne Grčke uključivale su ispaljive profile obe pretpostavljene roditeljske vrste (*A. alba* and *A. cephalonica*), gde su se učestalosti profila klinalno menjale po gradijentu georgrafske širine. Uzimajući u obzir da je jedan od pokazatelja prisustva hibridne zone klinalno variranje svih ili većeg broja karaktera lociranih na istom području, dobijena geografska distribucija isparljivih entiteta podržava hipotezu da su ispitivane populacije *A.* x *borisii-regis* sekundarnog porekla nastale hibridizacijom *A. alba* i *A. cephalonica*.

Ključne reči:
Abies x *borisii-regis*, Rodopi, hedspejs, isparljiva jedinjenja četina, hemodiverzitet

Introduction

Conifers of the genus *Abies* Mill. (Pinaceae) play an important ecological role in forest ecosystems of the northern hemisphere (Liepelt et al., 2010). Most of the *Abies* species are in the temperate zone, with a few of them on high mountains in subtropical areas (Xiang et al., 2007). The Mediterranean area is considered as one of the *Abies* distribution centers with about eight species depending on particular classification scheme. Mediterranean *Abies* spp. are weakly reproductively isolated and relatively easily hybridize in natural and artificial conditions as shown by crossability studies (Kormutak, 1985; 2004; Moulalis, 1986).

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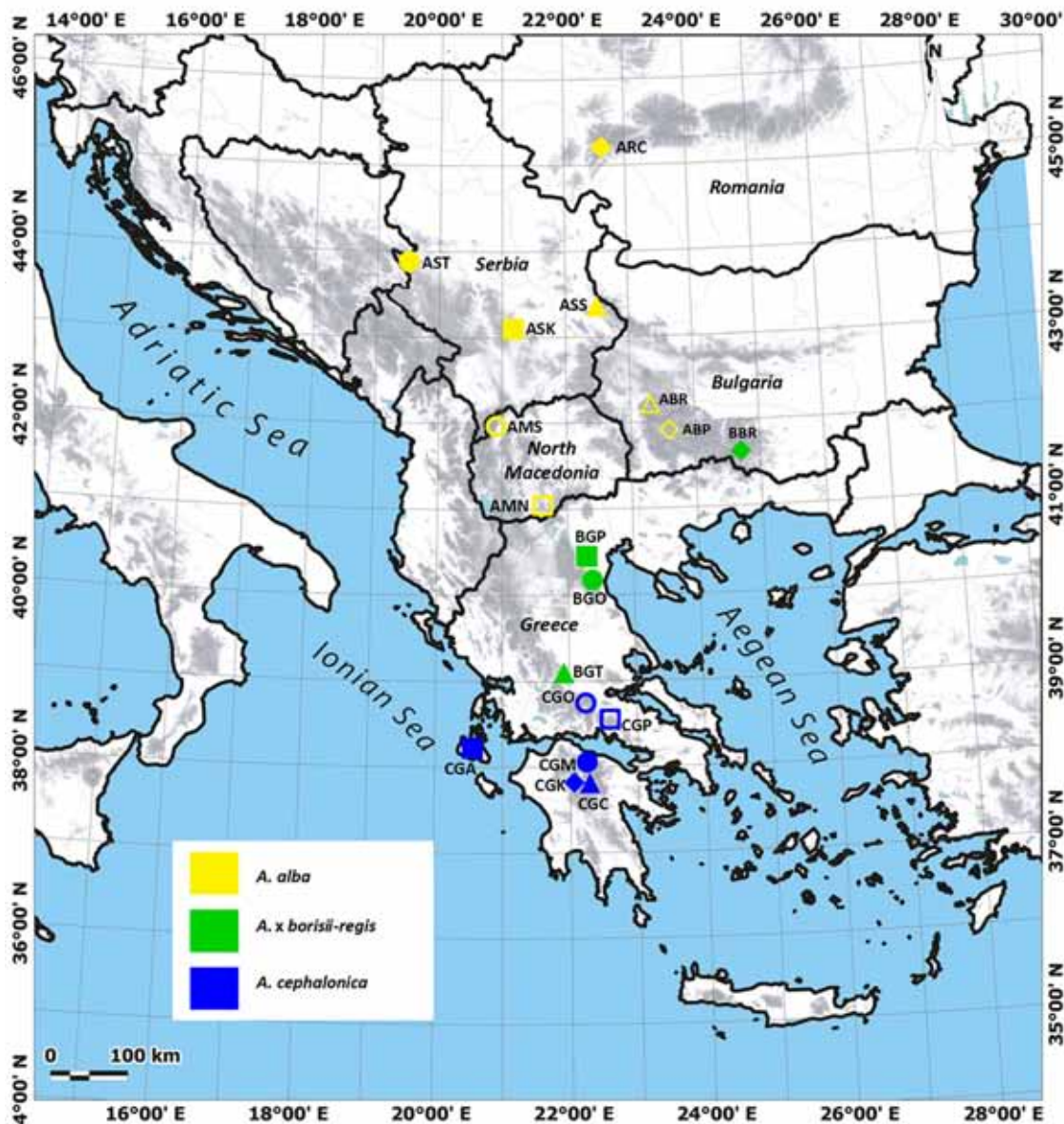*Abies x borisii-regis* from the central Rhodopes

Fig. 1. Geographical position of analyzed populations. For the description of the location and habitat conditions of the populations, cf. Tab. 1.

On the Balkan Peninsula, there are two native species of the section *Abies*: *A. alba* Mill. (Silver fir), widely distributed across Central Europe and some parts of Southern and Eastern Europe, and *A. cephalonica* Loudon (Greek fir), endemic to the mountain massifs of central and southern Greece (Panetsos, 1975). Except them, *A.* x *borisii-regis* Matff. (King Boris fir) was described from the central Rhodopes in Bulgaria by Mattfeld (1926) as a hybrid between *A. alba* and *A. cephalonica*. Subsequently, Turrill (1937) considered as equally possible that King Boris fir is an ancient lineage from which *A. alba* diversified towards the north and *A. cephalonica* towards the south. In addition, there

are a few more mutually exclusive hypotheses about its origin (Krajmerová et al., 2015). Nevertheless, in most previous classifications, *A.* x *borisii-regis* has the status of a hybrid between *A. alba* and *A. cephalonica* (Chater, 1964; Christensen, 1986; Christensen, 1997). Namely, it is considered that *A. alba* and *A. cephalonica* have experienced secondary contact in the refuges during periods of glacial expansion in Pleistocene. These events probably led to the exchange of genetic material between these two species in the glacial refugia which contributed to appearance of *A.* x *borisii-regis* (Liepelt et al., 2010).

The reports of *A.* x *borisii-regis* distribution range

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are highly controversial: according to one concept *A. x borisii-regis* appears parapatrically along with the putative parent species, i.e. in its northern distribution with *A. alba* and in southern with *A. cephalonica* (Jalas & Suominen, 1973; Christensen, 1997; Andreev, 1992). Thus, it occurs in southern Albania, almost the whole Greece (except the Cephalonia Island and the southern half of the Peloponnese), the southernmost parts of Northern Macedonia and across southwestern and southern Bulgaria (Jalas & Suominen, 1973). On the other hand, based on the Atlas of the Word's Conifers (Farjon & Filer, 2013), *A. alba*, *A. x borisii-regis*, and *A. cephalonica* are mostly allopatric, i.e., King Boris Fir is distributed in southern Albania, northern and northeastern Greece, and Rhodope Mt. in southern Bulgaria. Finally, Fady (1993) considered only the populations from Pindos Mt. and central Greece as *A. x borisii-regis*.

The usefulness of terpenes as volatile chemophenetic markers has been addressed in detail by Hanover (1992), who emphasized their importance in research of biodiversity, geographic variability, evolution, and systematics, particularly in studies of conifer order Pinales. Static headspace (HS) extraction is a common technique for analyzing volatile organic compounds, enabling the analyst to study a variety of sample matrices while avoiding the costly and time-consuming preparation involved with traditional gas chromatography (Kolb & Ettre, 2006). The advantage of combining of HS and GC-MS/FID (gas chromatography-mass spectrometry/flame ionization detection) methods in rapid and effective extraction and identification of highly volatile compounds as well as for chemotyping conifers has already been reported (Mitić et al., 2020; Nikolić et al., 2021).

In our previous study, seventeen native fir populations from the presumed zones of natural hybridization in the Balkan Peninsula were analyzed using HS needle volatiles as chemophenetic markers (Nikolić et al., 2021). *Abies alba* and *A. cephalonica* were characterized by distinct volatile profiles, while three populations from northern and central Greece identified as *A. x borisii-regis* shared the volatile profiles with both of the supposed parent species (Nikolić et al., 2021). Although King Boris fir was described from the central Rhodopes in Bulgaria (Mattfeld, 1926), recent molecular study did not provide evidence on hybrid origin of any Bulgarian fir populations including populations from this mountain massif (Krajmerová et al., 2015). Therefore, the aim of the present study was to shed more light on chemotaxonomic status of controversial Bulgarian fir populations. Hence, in this article we presented new results for one population of *A. x borisii-regis* from the southern

Bulgaria (the central Rhodopes) regarding diversity of HS needle volatiles. For multivariate statistical analyses (canonical discriminate analysis and agglomerative hierarchical clustering), in addition to the results obtained in this study, we used our previously published data relating to the broader transition zone between *A. alba* and *A. cephalonica* (Nikolić et al., 2021). In this way, HS needle volatiles of 18 populations of three *Abies* taxa from the Balkan Peninsula have been analyzed for the first time (**Fig. 1**).

Materials and Methods

Plant material

Plant material of *A. x borisii-regis* Mattf. was collected in the southern Bulgaria (the central Rhodopes, Pamporovo). Three-year-old needles were sampled during September 2021 from 14 individuals, which were randomly selected and distant at least 30 m. Plant material, deposited in labeled polyethylene bags (sample plot, date of collection, and locality), was immediately transferred to a freezer and stored at -20 °C prior to further analysis. Voucher specimen was deposited in the "Herbarium Moesiacum Niš" (HMN) of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš under the acquisition number 14389. Locations of the presently studied population, as well as 17 populations previously analyzed by Nikolić et al. (2021) are presented in **Fig. 1**. The corresponding geographic and geologic data, date of collection, voucher information as well as the number of studied individuals for every population are listed in the **Tab. 1**.

Headspace isolation

Three-year-old needles of every individual tree were cut into pieces of 2-3 mm length. Five hundred milligrams of chopped plant material was placed into 20-mL headspace vials and 1 mL of distilled water was added in each. The prepared vials were placed into a tray for further automated procedure. Each sample was heated at 80 °C for 20 minutes with the following program: shaking for 5 seconds, pausing for 2 seconds. After equilibration, the gas vapor of HS volatiles above the aliquot of volatiles was sampled with a gas syringe and subsequently injected into a GC analyzer.

GC-MS/FID analyses

Qualitative analysis of the HS volatiles was performed by Gas Chromatography-Mass Spectrometry (GC-MS), while the quantitative data were obtained by Gas Chromatography-Flame Ionization Detector (GC-FID) analysis. Samples

Table 1. Location and habitat description of the selected populations of three *Abies* taxa

Taxon	Locality	Population code	Latitude (N)	Longitude (E)	Altitude (m.a.s.l.)	Substratum	Number of individuals analyzed	Date of collection	Number of vauchers
A. alba	Romania, Southern Carpathians, Transfăgărăşan	ARC	45°23'30"	24°37'56"	957	Silicate	5	August, 2018	13950 HMN
A. alba	Serbia, Mt. Tara, Predov Krst	AST	40°11'47"	22°10'48"	1096	Limestone	15	June, 2018	13949 HMN
A. alba	Serbia, Mt. Kopaonik, Kadijevac	ASK	43°19'43"	20°45'41"	1350	Silicate	15	June, 2017	13946 HMN
A. alba	Serbia, Mt. Stara planina, Arbinje	ASS	43°18'25"	22°47'18"	1295	Silicate	15	August, 2018	13944 HMN
A. alba	North Macedonia, Mt. Šar-planina, Lešnica	AMS	42° 1'17"	20°47'04"	1505	Limestone	15	August, 2017	13945 HMN
A. alba	North Macedonia, Mt. Nidže	AMN	40°58'54"	21°47'31"	1900	Silicate	15	August, 2018	13948 HMN
A. alba	Bulgaria, Mt. Pirin, above Bansko	ABP		23°28'33"	1290	Silicate	15	October, 2018	13942 HMN
A. alba	Bulgaria, Mt. Rila, above Rila Monastery	ABR	42°09'17"	23°24'49"	1536	Silicate	15	October, 2018	13947 HMN
A. x borisii-regis	Bulgaria, Mt. Rhodopes, Pamporovo	BBR	41°39'01"	24°41'40"	1620	Silicate	14	September, 2020	14389 HMN
A. x borisii-regis	Greece, Mt. Pieria, Fteri	BGP	40°11'48"	22°11'72"	921	Silicate	15	October, 2018	13943 HMN
A. x borisii-regis	Greece, Mt. Olympus, above Leptokarya	BGO	40° 01'54"	22°30'25"	640	Limestone	15	October, 2018	13951 HMN
A. x borisii-regis	Greece, Mt. Tymfristos, Sellos	BGT	38°54'33"	21°54'36"	971	Limestone	15	September, 2019	13997 HMN
A. cephalonica	Greece, Mt. Oiti	CGO	38°44'15"	22°22'54"	895	Limestone	15	September, 2019	14002 HMN
A. cephalonica	Greece, Mt. Parnassos, Pleiades	CGP	38°33'06"	22°34'10"	1660	Limestone	15	September, 2019	14001 HMN
A. cephalonica	Greece, Peloponnese, Mega Spilaio Monastery	CGM	38°05'17"	22°10'23"	860	Limestone	15	September, 2019	14000 HMN
A. cephalonica	Greece, Peloponnese, Mt. Chelmos, Kalavryta	CGK	38°00'41"	22°09'52"	1368	Limestone	15	September, 2019	13998 HMN
A. cephalonica	Greece, Peloponnese, Mt. Chelmos	CGC	38°00'56"	22°10'57"	1550	Limestone	15	September, 2019	13999 HMN
A. cephalonica	Greece, Cephalonia, Mt. Ainos	CGA	38°09'37"	20°37'13"	1005	Limestone	15	July, 2019	13996 HMN

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were analyzed on Agilent Technologies 7890B GC equipped with a fused silica capillary column (HP-5MS, 250 μm×25 m, film thickness 0.25 μm, Agilent Technologies, Santa Clara, CA, USA) and coupled with a 7890A flame ionization detector (FID) and 7000B MS/MS spectrometer (operating in MS1 scan mode) from the same company. The GC was operated under the following conditions: injector temperature 250 °C; GC-MS interface temperature 300 °C; oven temperature programmed from 50 °C for 2.25 minutes, then to 200 °C at 4 °C/minute (carrier gas He, 1.0 mL/min, constant flow mode). MS conditions were as follows: ionization voltage of 70 eV; acquisition mass range 40-440; scan time 0.32 seconds. HS volatiles were identified from TIC (Total Ion Chromatogram) by comparison of their linear retention indices relative to C_8-C_{20} *n*-alkanes recorded on the same column/temperature program with literature values (NIST MS Search 2.0; NIST Chemistry WebBook SRD69; Adams, 2007) and their mass spectra with those of standards from databases (Wiley 6, NIST02, Adams) by the application of the AMDIS software (the Automated Mass Spectral Deconvolution and Identification System, Ver. 2.7, distributed within the software package for 7890-7000 BGC-MS/MS triple quadrupole system). The percentage composition of the HS volatiles was computed from the GC-FID peak areas without any corrections.

Statistical analyses

Statistical data processing was carried out by STATISTICA 8 software (Statsoft, Inc., Tulsa, OK, USA). Statistical matrices included HS volatiles that were detected in the content ≥0.5% in at least one of the analyzed taxa (12 components in total: santene, tricyclene, α-pinene, camphene, β-pinene, myrcene, α-phellandrene, limonene+β-phellandrene, terpinolene, borneol, bornyl acetate, (*E*)-caryophyllene). Multivariate analysis included canonical discriminant analysis (CDA) and agglomerative hierarchical clustering (AHC). Overall differences between the compared groups are presented by Mahalanobis distances. The calculated matrix distance was used for Agglomerative Hierarchical Clustering (AHC), using the Ward's method.

Results and discussion

Variability of HS needle volatiles

GC-MS/FID analyses of HS needle volatiles in *A.* x *borisii-regis* population from the central Rhodopes revealed the presence of 34 compounds (**Tab. 2**). The experimental and literary retention indices, identification methods, and classes to which

identified compounds belong are also presented in **Tab. 2**.

As the most volatile compounds, monoterpene hydrocarbons represented the major compound class (95.3%), while contents of oxygenated monoterpenes and sesquiterpene hydrocarbons were significantly lower (2.2 and 2.4%, respectively). Further, the phytochemical profile of the main HS volatiles for studied population could be presented as follows:

β-pinene >>> α-pinene > camphene > limonene+β-phellandrene

(where, based on Petrakis et al. (2001), =, >, >>, and >>> represent difference of 0.1-1.0%, 1.1-5.0%, 5.1-15.0%, and more than 15.1%, resp.).

According to Nikolić et al. (2021), the phytochemical profiles of the dominant HS volatiles of *A. alba*, *A.* x *borisii-regis*, and *A. cephalonica* were:

A. alba: β-pinene >> limonene+β-phellandrene > camphene > α-pinene

A. x *borisii-regis*: β-pinene >> α-pinene >> camphene > limonene+β-phellandrene

A. cephalonica: β-pinene > α-pinene >>> camphene > limonene+β-phellandrene

Thus, presently reported volatile profile of Bulgarian fir population from the central Rhodopes fits well with described profile of Greek *A.* x *borisii-regis* populations.

Multivariate analyses (CDA and AHC) of HS needle volatiles at the population level

The CDA based on 259 individuals from 18 populations of *A. alba*, *A.* x *borisii-regis*, and *A. cephalonica* showed that the first two canonical axes participated in 62.9% of the total discrimination, of which the first axis (CA1) accounted for 44.5% (**Fig. 2** and **Tab. 3**). Four compounds, i.e. β-pinene, limonene+β-phellandrene, camphene, and α-pinene had significant impact on both axes, while bornyl acetate significantly affected only the CA2 (**Tab. 3**). Based on the obtained scatterplot (**Fig. 2**), all populations of *A. alba* showed positive values for the CA1 (yellow ellipse), while *A. cephalonica* populations formed a group at the negative part of CA1 (blue ellipse). According to the results of the post-hoc test that was done in the study of Nikolić et al. (2021), the "alba" profile was characterized by higher content of limonene+β-phellandrene and camphene and lower content of α-pinene compared to the second profile; *vice versa* the "cephalonica" profile was characterized by lower amount of limonene+β-phellandrene and camphene and higher

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Table 2. Relative percentage composition of HS needle volatiles of Bulgarian *A. x borisii-regis* population from the central Rhodopes

Entry	Compounds	RI[1]	LI[2]	Content (%)[3] n=14	Class of compounds	Identification method
1.	Hexanal	801	801	tr	O	RI, MS
2.	(*2E)*-Hexenal	849	846	0.1±0.2	O	RI, MS
3.	(*3Z)*-Hexenol	850	850	tr	O	RI, MS
4.	Hexanol	866	863	tr	O	RI, MS
5.	Santene	884	884	0.5±0.2	MH	RI, MS
6.	Tricyclene	921	921	3.3±1.2	MH	RI, MS
7.	**α-Pinene**	934	932	**19.7±2.5**	MH	RI, MS
8.	**Camphene**	949	946	**16.3±4.2**	MH	RI, MS
9.	**β-Pinene**	980	974	**40.3±5.5**	MH	RI, MS
10.	Myrcene	990	988	1.2±0.3	MH	RI, MS
11.	α-Phellandrene	1004	1002	0.3±0.2	MH	RI, MS
12.	**Limonene+β-Phellandrene**	1029	1024/1025	**13.3±2.1**	MH	RI, MS
13.	2-Heptyl acetate	1040	1038	tr	O	RI, MS
14.	γ-Terpinene	1057	1054	tr	MH	RI, MS
15.	Terpinolene	1087	1086	0.4±0.1	MH	RI, MS
16.	2-Nonanone	1089	1087	tr	O	RI, MS
17.	Borneol	1166	1165	1.0±0.9	MO	RI, MS
18.	Linalyl acetate	1254	1254	tr	MO	RI, MS
19.	Bornyl acetate	1287	1287	1.2±1.0	MO	RI, MS
20.	α-Longipinene	1353	1350	0.1±0.1	SH	RI, MS
21.	α-Ylangene	1373	1373	tr	SH	RI, MS
22.	α-Copaene	1377	1374	tr	SH	RI, MS
23.	Sibirene	1404	1400	tr	SH	RI, MS
24.	Longifolene	1409	1407	tr	SH	RI, MS
25.	(*E*)-Caryophyllene	1420	1417	1.6±1.2	SH	RI, MS
26.	Himachala-2,4-diene	1429	1429[2a]	0.1±0.2	SH	RI, MS
27.	Humulene	1456	1452	0.4±0.3	SH	RI, MS
28.	β-Selinene	1490	1489	tr	SH	RI, MS
29.	δ-Selinene	1494	1492	0.1±0.1	SH	RI, MS
30.	α-Selinene	1498	1498	tr	SH	RI, MS
31.	β-Himachalene	1503	1500	tr	SH	RI, MS
32.	δ-Amorphene	1509	1511	tr	SH	RI, MS
33.	γ-Cadinene	1516	1513	tr	SH	RI, MS
34.	δ-Cadinene	1525	1522	tr	SH	RI, MS
	Total			100.0±0.2		
	Monoterpene hydrocarbons (MH)			95.3±1.9		
	Oxygenated monoterpenes (MO)			2.2±1.8		
	Sesquiterpene hydrocarbons (SH)			2.4±1.8		
	Others (O)			0.2±0.2		

[1]RI: Experimental linear retention indices relative to C_8-C_{20} alkanes. [2]LI:Literature indices-Adams' retention indices and [2a]according to NIST data base. [3]Contents are given as percentages (mean±standard deviation) of the total compounds content; *n*: the number of analyzed individuals; tr - trace<0.05%; (-) not detected compounds; the most abundant HS volatiles are in boldface.

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amount of α-pinene compared to the "alba" profile.

Accordingly, eastern *A. alba* lineage and *A. cephalonica* may be undoubtedly characterized by volatile markers, while populations of *A.* x *borisii-regis* were positioned in the intermediate position. Specifically, all four populations identified as *A.* x *borisii-regis* included volatile profiles of both supposed parent species, whereby profile frequencies changed clinally along the latitudinal gradient (**Fig. 2**). In the northernmost population of *A.* x *borisii-regis* from southern Bulgaria (Rhodopes Mt. - BBR) most individuals belonged to the "alba" profile (the largest number of green diamonds is situated within the yellow ellipse), while individuals from two northern Greece populations were more evenly distributed on both sides of CA1. Nevertheless, in a slightly more northern population (Pieria Mt. - BGP) a larger number of individuals belonged to the "alba" profile (green squares more abundant within the yellow ellipse), while individuals from a somewhat southern population (Olympus Mt. - BGO) were evenly distributed on both sides of CA1, indicating approximately equal frequencies of both profiles (green circles with approximately equal representation within both ellipses). Finally, in the southernmost *A.* x *borisii-regis* population from the central Greece (Tymfristos Mt. - BGT) most individuals were located in the negative part of CA1, closer to the "cephalonica" profile (the largest number of green triangles is situated within

Table 3. Standardized coefficients for the first two canonical axes (CA) of variation in 12 needle volatiles from the discriminant functional analysis of 18 a priori groups. The most significant coefficients are in boldface

Variables	CA1	CA2
Santene	-0.105	-0.164
Tricyclene	0.827	-0.905
α-Pinene	**1.123**	**1.526**
Camphene	**2.193**	**2.934**
β-Pinene	**4.207**	**2.859**
Myrcene	0.063	0.056
α-Phellandrene	-0.089	0.203
Limonene+β-Phellandrene	**3.690**	**2.987**
Terpinolene	0.066	-0.177
Borneol	0.468	-0.712
Bornyl acetate	0.610	**1.239**
(*E*)-Caryophyllene	0.730	0.436
Eigenvalue	4.197	1.729
% explained variation	0.445	0.629

the blue ellipse). Therefore, all four studied *A.* x *borisii-regis* populations included both the *A. alba* and *A. cephalonica* volatile profiles, but distributed along a latitudinal gradient connecting the ranges of presumed parent species, which confirms the hypothesis proposed in our previous study (Nikolić et al., 2021). Considering that one of the indicators of the presence of a hybrid zone is the clinical variation of all or a larger number of characters located in the same area, the observed geographic distribution of volatile entities supports the hypothesis that studied *A.* x *borisii-regis* populations are of secondary origin due to hybridization between *A. alba* and *A. cephalonica*. In addition, clinal (north-south) pattern in the distribution of morpho-anatomical (Mitsopoulos & Panetsos, 1987) and genetic traits (Krajmerová et al., 2015) over the *A.* x *borisii-regis* range was previously reported.

The dendrogram obtained by AHC analysis has also shown a tendency to divide analyzed fir populations in similar way (**Fig. 3**). Namely, populations of *A. alba* and *A. cephalonica* were divided in two separate clusters, while populations of *A.* x *borisii-regis* appeared heterogeneous. Actually, the northernmost population of *A.* x *borisii-*
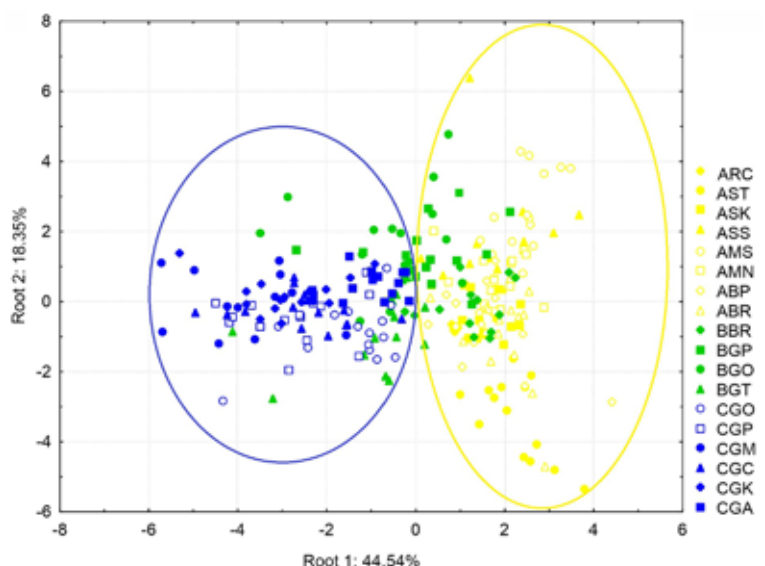

Fig. 2. Canonical discriminant analysis (CDA) based on contents of 12 HS needle volatiles isolated from 259 individuals of 18 populations. Abbreviations and symbols refer to populations as indicated in Tab. 1. and Fig 1. Abbreviation colors: yellow – *A. alba*, green – *A. x borisii-regis*, and blue – *A. cephalonica*. The yellow and blue ellipses encompass all individuals of supposed parent species.
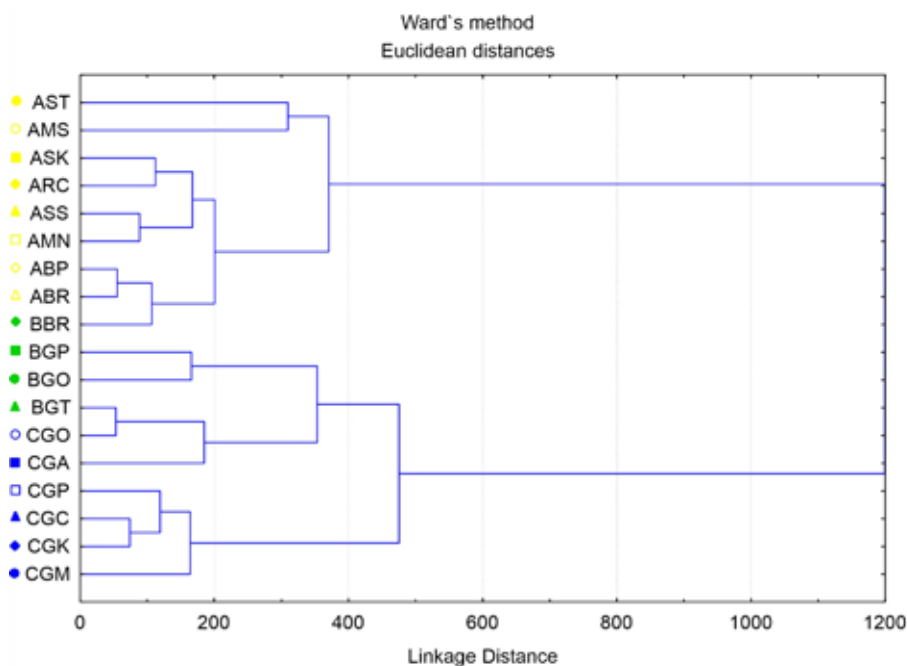
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Fig. 3. Dendrogram obtained by agglomerative hierarchical clustering (AHC) of 18 populations based on 12 HS needle volatiles. Abbreviations and symbols refer to populations as indicated in Tab. 1. and Fig 1. Abbreviation colors: yellow - *A. alba*, green - *A. x borisii-regis*, and blue - *A. cephalonica*

regis from southern Bulgaria (BBR) fitted within the "alba" and all Greek populations of *A. x borisii-regis* (BGP, BGO, and BGT) within the "cephalonica" cluster. Furthermore, the northernmost (BBR) and southernmost (BGT) population of *A. x borisii-regis* showed the greatest resemblance to the geographically closest populations of the parent species: southwestern Bulgarian populations of *A. alba* (ABR and ABP) and population of *A. cephalonica* from central Greece (CGO), respectively (**Fig. 3**).

The history of fir populations from Rhodope Mt. is largely unknown; fluctuating presence of fir pollen was recorded in Rhodope sites since the end of the Pleniglacial until the early Holocene and an expansion occurred only after 8500 BP (Terhürne-Berson et al., 2004). Although King Boris fir was described from the central Rhodopes (Mattfeld, 1926), recent molecular study did not provide evidence on hybrid origin of any of 10 analyzed populations from this mountain massif, based on maternally inherited mitochondrial (mtDNA) markers (Krajmerová et al., 2015). Moreover, these authors found exclusively *A. alba* mitotype in all fir populations from Bulgaria, North Macedonia, and northern Greece, while only two populations from central Greece (Tymfrostos and Rentina Mts.) were mixed and contained predominantly the *A. cephalonica* mitotype, with a lower proportion of the *A. alba* mitotype. Even though Bella et al. (2014)

analyzed the same mtDNA marker (nad5-4) as in the study of (Krajmerová et al., 2015), they found a mixture of maternal lineages (mitotypes of both parent species) in two northern Greek populations (Pieria and Pindos Mts.). Nevertheless, the second study did not include any of fir populations from central Greece, Bulgaria, or North Macedonia. According to phytochemical markers selected in the present study, presence of both volatile profiles was recorded in a somewhat wider geographical area. i.e. four populations from the southern Bulgaria, northern and central Greece, where the northernmost population contained predominantly *A. alba* and the southernmost *A. cephalonica* profile (**Fig. 2**). Thus, based on this concept, *A. alba* and *A. cephalonica* were co-occurring at least in the southern Bulgaria, northern and central Greece, suggesting that this region may have served as a refugium for *A. alba* during the Pleistocene glacial cycles. Bearing in mind several proposed distribution concepts of King Boris fir, our results are mostly in agreement with the concept given in the Atlas of the Word's Conifers (Farjon & Filer, 2013) with the addition of central Greece.

Conclusions

GC-MS/FID analyses of HS needle volatiles in population *A. x borisii-regis* from the southern Bulgaria (the central Rhodopes) revealed presence

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of 34 compounds. The major HS volatiles were β-pinene (40.3%), α-pinene (19.7%), camphene (16.3%) and limonene+β-phellandrene (13.3%). The presently reported phytochemical profile of the main HS volatiles fits well with previously described profile of Greek *A.* x *borisii-regis* populations. Based on multivariate statistical analyses, all four populations identified as *A.* x *borisii-regis* from southern Bulgaria, northern and central Greece included volatile profiles of both supposed parent species, where profile frequencies changed clinally along the latitudinal gradient connecting the ranges of parent species. Thus, based on HS needle volatiles, *A. alba* and *A. cephalonica* were co-occurring at least in the southern Bulgaria, northern and central Greece, suggesting that this region may have served as a refugium for *A. alba* during the Pleistocene glacial cycles. As a natural hybrid, populations of King Boris fir can be interesting from the point of view of commercial forestry for the reason that offer the possibility to cope with the predicted forest decline in Southern Europe as a result of climate change and can be used for reforestation.

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