

Taxonomic position of *Abies equi-trojani* on the basis of needle characters by comparison with different fir species

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Abstract: The needles from 25 trees of *Abies equi-trojani* from Kazdağı (Mount Ida) were compared with those from four populations of *A. alba*, two of *A. cephalonica*, and three of each of *A. ×borisii-regis*, *A. bornmuelleriana*, and *A. nordmanniana*, each represented by 29–31 individuals. Thirty-nine morphological and anatomical needle characters (26 main and 13 estimated ones) were examined biometrically to verify the systematic position of *A. equi-trojani*. To evaluate the relationships among taxa, Student's t-test for independent samples, the Mann–Whitney U test, and discrimination and clustering analyses were applied. The differences between taxa were statistically significant for several characters, but the data ranges generally overlapped between species. Morphological and anatomical characters of the needle allowed to distinguish between *A. equi-trojani*, *A. alba*, *A. cephalonica*, *A. ×borisii-regis*, *A. bornmuelleriana*, and *A. nordmanniana* when used together, but no single character applied separately could distinguish between species. A close relationship of *A. equi-trojani* to *A. bornmuelleriana* and to *A. nordmanniana* detected based on the needle characters supported the infraspecific systematic position of these three taxa.

Key words: Multivariate analyses, needle variation, plant variation, taxonomy

1. Introduction

The name '*Abies equi-trojani*' was proposed by Paul Sintenis and Paul Ascherson on the herbarium label (Sintenis, 1883, pl. exicc. Iter trojanum, No 523) for the material collected from Kazdağı (Mount Ida), but without a diagnosis, which had been made by Boissier (1884), who treated the specimens collected by Sintenis as *A. pectinata* DC. var. *equi-trojani* Asch. & Sint. ex Boiss. Shortly thereafter, this taxon was included as a variety of *A. nordmanniana* Spach by Guinier and Maire (1908): *A. nordmanniana* var. *equi-trojani* (Asch. & Sint. ex Boiss.) Guinier & Maire; and more recently as a subspecies of silver fir: *A. alba* Mill. subsp. *equi-trojani* (Asch. & Sint. ex Boiss.) Asch. & Graebn. (for details see Yalçırık, 1974).

The systematic position of *A. equi-trojani* has been disputed several times due to its intermediate position between *A. cephalonica* Loudon and *A. nordmanniana* with respect to the morphological characters of cones, types of needle apex, position of resin canals in the needle, pollen grain morphology, and wood anatomy (Mattfeld, 1925; Aytug, 1959; Liu, 1971; Yalçırık, 1974; Bağcı and Babaç, 2003; Kaya et al., 2008; Kurt et al., 2016; also Aytug PhD

thesis, İstanbul University, Faculty of Forestry, 1958). The marginal position of the resin canals in the needles from lower branches and the median position of the needles of cone-bearing branches were a basis for inclusion of *A. equi-trojani* in *A. cephalonica* var. *graeca* (Fraas) Liu, which occurs in the Parnassus, whereas *A. cephalonica* var. *cephalonica* overlaps with all other populations of this species in Greece (Liu, 1971). However, detailed studies on *A. cephalonica* using both morphological characters and genetic markers indicated that the varieties proposed by Liu (1971) are indistinguishable (Panetsos, 1975; Mitsopoulos and Panetsos, 1987; Panetsos, 1992). *Abies equi-trojani*, on the other hand, appeared distant from *A. cephalonica* biochemically (Mitsopoulos and Panetsos, 1987) and genetically (Scaltsoyiannes et al., 1999; Liepelt et al., 2010). Lately, *A. ×olcayana* C. Ata & N. Mervev, intermediate between *A. equi-trojani* and *A. bornmuelleriana* Mattf., has been described from Çataldağ (Ata and Mervev, 1981, 1987).

The subspecific taxonomic status of *A. nordmanniana* subsp. *equi-trojani* (Asch. & Sint. ex Boiss.) that Coode and Cullen proposed in *Flora of Turkey* (Coode and

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Cullen, 1965; Cullen and Coode, 1965) has been accepted in more recent works (Farjon and Rushforth, 1989; Farjon, 2010), but it has also been treated as an independent species (Schütt, 1991; Yaltırık, 1993; Veliöglu et al., 1998; Kaya et al., 2008; Debreczy and Rácz, 2011; Kurt et al., 2016). The nomenclatural discrepancies were a reason for several analyses of the morphological and anatomical characteristics, as well as the genetic diversity and differentiation of this taxon. *Abies equi-trojani* has been compared to various provenances of *A. cephalonica*, *A. bornmuelleriana*, and *A. nordmanniana*, and to *A. cilicica* (Antoine & Kotschy) Carrière, *A. alba*, and *A. ×borisii-regis* Mattf. (Mitsopoulos and Panetsos, 1987; Panetsos, 1992; Scaltsoyiannes et al., 1999; Bağcı and Babaç, 2003; Liepelt et al., 2010; Kurt et al., 2016) using different morphological and genetic markers.

The relationships among the firs of the Balkans and North Anatolia are generally better detectable when using genetic markers rather than morphological characteristics (Scaltsoyiannes et al., 1999; Kaya et al., 2008; Liepelt et al., 2010). Within the latter set, cone length and diameter, cone scale shape, cone bract shape, and the level of insertion among the cone scales are recognized as essential to distinguish between species (Yaltırık, 1993; Bağcı and Babaç, 2003; Farjon, 2010; Kurt et al., 2016). From the needle characteristics, the arrangement of needles on the branchlet, needle length, needle width, needle thickness, position of the resin canals, and form of the needle apex are utilized in the species descriptions and in the keys to distinguish between them (Chater, 1964; Coode and Cullen, 1965; Liu, 1971; Yaltırık, 1993; Christensen, 1997; Farjon, 2010). These characters were also used in a comparative study between the *Abies* species that occur in Turkey (Bağcı and Babaç, 2003). Multivariate analyses done by these authors segregated only a part of the compared populations to the taxa correctly and, not surprisingly, *A. equi-trojani* appeared close to *A. bornmuelleriana* but also intermingled among samples of *A. cilicica* (Bağcı and Babaç, 2003).

Biometric analysis of a much broader set of needle morphological and anatomical characters distinguished clearly between *A. cilicica* subsp. *cilicica* and *A. cilicica* subsp. *isaurica* Coode & Cullen, and even detected further geographic differentiation of the typical subspecies (Boratyńska et al., 2015). We expect that the same set of needle characters could allow us to distinguish *A. equi-trojani* from *A. cephalonica*, *A. ×borisii-regis*, *A. alba*, *A. bornmuelleriana*, and *A. nordmanniana* and clarify reciprocal relations among these species. We also assumed that the detailed needle characteristics of the Balkan and North Anatolian *Abies* species could provide a set of data that allows for better determination of the subfossil materials, where needles constitute the most frequently found remains (Palamarev, 1989; Kovar-Eder et al., 2006).

We hypothesized that the position of the fir from Kazdağı would appear intermediate between Balkan *A. cephalonica* and *A. ×borisii-regis* and the Anatolian *A. bornmuelleriana*, while being more distant from the Carpathian *A. alba* and East Anatolian-Caucasian *A. nordmanniana*. Verification of this hypothesis is the aim of the study, using a biometric comparison of these taxa based on the needle morphological and anatomical characteristics.

2. Materials and methods

2.1. Studied species and plant material

The systematic status of the Balkan-Anatolian fir taxa was unstable (see above). For that reason, the examined firs are treated here provisionally as independent species:

- *A. equi-trojani* (Asch. & Sint. ex Boiss.) Mattf. = *A. nordmanniana* subsp. *equi-trojani* sensu Coode and Cullen (1965) and Farjon (2010)

- *A. bornmuelleriana* Mattf. = *A. nordmanniana* subsp. *bornmuelleriana* sensu Coode and Cullen (1965) and Farjon (2010)

- *A. nordmanniana* Spach = *A. nordmanniana* subsp. *nordmanniana* sensu Coode and Cullen (1965) and Farjon (2010)

We analyzed *A. equi-trojani* from Kazdağı, comparing it with *A. alba* from the northeastern limit of the geographic range of this species, away from the possible influence of the *Abies* species from populations hypothetically representing *A. ×borisii-regis* from the mountains of Bulgaria and populations of *A. cephalonica*, *A. nordmanniana*, and *A. bornmuelleriana* (Table 1). Each population was represented by approximately 30 adult trees, except for *A. equi-trojani*, of which only 25 trees were sampled. From every individual tree, 10 needles were collected from the central part of the 2-year-old yearly increment of the lateral branchlet, exposed to the south, approximately 2–7 m above ground level. The plant material was conserved in 70% alcohol immediately after collection and preserved until preparation and measurements at a temperature of –20 °C. In total, 4750 needles were examined biometrically, representing 475 individual trees. Five needles from every tree were analyzed morphologically, and another five were used to prepare cross-sections for taking anatomical measurements. The set of characters used, as well as the methods of preparation and measurement, were adopted from investigations of the West Mediterranean (Sękiewicz et al., 2013) and Turkish firs (Bağcı and Babaç, 2003; Boratyńska et al., 2015) after verification of their usefulness. Finally, 26 main and 13 estimated characters were analyzed (Table 2; Figure 1).

Table 1. Geographic data for studied fir populations.

Code	Location	N	Longitude E [°]	Latitude N [°]	Altitude [m]
AET	Turkey, Kaz Dağı	25	26.87	39.72	1430
AA1	Poland, Świętokrzyskie Mountains, Suchedniów, Dolejów	30	20.74	51.07	310
AA2	Poland, Roztocze, Guciów	30	23.07	50.59	210
AA3	Ukraine, Carpathian Mountains, Kniaźdwór Nature Reserve	30	24.92	48.55	400
AA4	Ukraine, Carpathian Mountains, Czernivcy	30	26.18	48.02	400
ABR1	Bulgaria, Rila, above Rilski Monastyr	30	23.40	42.15	1400
ABR2	Bulgaria, Rila, Borovec	31	23.59	42.26	1400
ABR3	Bulgaria, Pirin, above Bansko	29	23.47	41.81	1250
ACE1	Greece, Euboea, Mount Dirfys	30	23.84	38.62	1600
ACE2	Greece, Kefalonia Island, Ainos Oros	30	20.62	38.16	1100
ANN1	Turkey, Kalkanlı Dağları, Zigana	29	39.35	40.60	1400
ANN2	Turkey, Parmak Dağı, Artvin	30	41.77	41.15	1660
ANN3	Turkey, Şavşat	30	42.43	41.23	1700
ANB1	Turkey, Uludağ	30	29.13	40.10	1920
ANB2	Turkey, Ilgaz Dağları	30	33.73	41.07	1870
ANB3	Turkey, Küre Dağları	31	34.84	41.64	1230

AET – *Abies equi-trojani*, AA – *A. alba*, ACE – *A. cephalonica*, ANN – *A. nordmanniana*, ANB – *A. bornmuelleriana*, ABR – *A. ×borisii-regis*, N – number of individuals

2.2. Statistical treatment

The normality of the frequency distribution of every measured character was verified using the Shapiro–Wilk *W* test and the homoscedasticity of variances of the data were compared using the Brown–Forsythe test. The data describing evaluated characters concerning particular variants of presence of stomata on the adaxial needle side (LSU), forms of the needle apex (TA), and position of the resin canals (LC) were converted to percentages. Additionally, the data were standardized before multivariate statistical analyses to avoid the possible influence of different character types (Zar, 1999) using STATISTICA v. 9.0 procedures (StatSoft PL).

Interactions between pairs of characters were verified with Pearson's correlation coefficient to detect possible redundant variables, with $r \geq |0.90|$. Basic statistics such as arithmetic means and coefficients of variation were calculated for each population and species to determine the range of their variation. To evaluate the significance of differences between studied fir species, Student's *t*-test (measured and ratio characters) and the Mann–Whitney *U* test (evaluated and characters with skewed frequency distribution) for independent samples were used.

Discrimination analysis (DA) was conducted on the proportions and directly measured characters were not taken into account in proportions. Extreme variables (NRU, NSU, RR, and SR), estimated variables (LSU, TA and LC variants, NML, NC, and CH), and those of single characters from every highly correlated pair were excluded from DA, which was finally conducted using 15 characters (A, NRL, NSL, MT, NS, VCS, RW_1–4, LMW, MC, ES, HS, CS). Discrimination allowed us to eliminate redundant characters and illustrate the relationships between taxa and populations (Sokal and Rohlf, 1997). Afterward, DA was used again to detect the correctness of including individuals into a particular species.

Agglomeration analysis on the same characters used in DA, per Ward's method on the shortest Euclidean distances and on the squares of the Mahalanobis distances, was applied to verify relationships detected with DA (Zar, 1999). Afterward, *K*-means cluster analysis (*K*-MCA) was applied as a supplement to agglomeration. *K*-MCA revealed the number of *K*-clusters that illustrated population differentiation optimally. To determine the optimal number of *K*-groups, a scree plot was used on the basis of Δd value minimization when adding the subsequent nodes

Table 2. Values of needle characters of *Abies equi-trojani* and P-values of t-test for independent samples and Mann–Whitney’s U test* when compared with studied fir species; significant values of P are bolded.

Character	Code	AET characteristics				P of t and/or U tests				
		M	Min	Max	V	AET/AA	AET/ABR	AET/ANB	AET/ANN	AET/ACE
Needle area [mm ²]	A	36.47	16.21	69.99	25.5	0.002	0.428	0.661	0.307	0.969
Needle perimeter [mm]	P	51.23	27.46	92.33	20.8	0.013	0.504	0.880	0.778	0.012
Needle length [mm]	L	23.79	12.89	40.62	18.7	0.064	0.238	0.837	0.402	0.000
Needle maximum width [mm]	MW	1.76	1.23	2.04	8.0	0.000	0.000	0.025	0.000	0.000
Needle width at 95% of its length [mm]	W_95	1.37	0.80	1.77	10.5	0.488	0.271	0.000	0.000	0.026
Needle width at 50% of its length [mm]	W_50	1.66	1.16	2.10	9.2	0.000	0.000	0.155	0.000	0.000
Distance from the basis to the needle maximum width [mm]	BD	12.43	6.40	20.50	21.9	0.670	0.041	0.901	0.496	0.040
Number of stoma rows on adaxial needle surface at the central part of needle	NRU	0.01	0.00	1.00		0.827	0.851	0.108	0.783	0.021
Number of stomata on 1 mm of central part of adaxial side of needle surface	NSU	0.00	0.00	0.00	0.00	0.998	0.997	0.931	0.997	0.016
Number of stoma rows on abaxial needle surface at the central part of needle	NRL	13.66	9.00	17.00	9.6	0.140	0.319	0.000	0.000	0.000
Number of stomata on 1 mm of central part of abaxial side of needle surface	NSL	9.39	7.50	11.50	6.6	0.000	0.182	0.004	0.013	0.244
Needle width on the cross-section [µm]	SW	1826.1	1306.3	2199.5	7.8	0.002	0.000	0.135	0.002	0.000
Needle thickness on the cross-section [µm]	ST	739.2	559.9	973.1	10.1	0.000	0.101	0.000	0.018	0.000
Width of endodermis tube [µm]	VCW	469.7	293.3	639.8	11.0	0.000	0.166	0.001	0.885	0.000
Height of endodermis tube [µm]	VCT	296.7	213.3	359.9	9.1	0.000	0.103	0.000	0.484	0.000
Number of mesophyll palisade layers	NML	1.57	1	2	9.5	0.005	0.126	0.710	0.538	0.000
Thickness of one of the mesophyll palisade layers [µm]	MT	77.9	51.4	107.0	8.6	0.036	0.356	0.007	0.000	0.000
Distance between vascular bundles [µm]	DV	39.3	4.3	77.0	27.0	0.000	0.000	0.000	0.000	0.635
Width of epidermal cell [µm]	EW	23.5	17.1	34.2	8.6	0.000	0.000	0.142	0.580	0.017
Height of epidermal cell [µm]	EH	21.5	15.0	25.7	9.0	0.000	0.058	0.000	0.001	0.000
Width of hypodermal cell [µm]	HW	19.6	13.9	27.11	8.9	0.000	0.001	0.158	0.043	0.115
Height of hypodermal cell [µm]	HH	20.0	12.8	25.7	9.1	0.000	0.106	0.339	0.039	0.000
Width of resin canal [µm]	WC	73.5	38.5	128.4	17.9	0.000	0.000	0.000	0.038	0.000
Height of resin canal [µm]	HC	69.7	30.0	124.1	19.6	0.000	0.000	0.000	0.043	0.000
Number of resin canals	NC	1.99	1	2	3.1	0.775	0.783	0.922	0.857	1.000
Continuity of hypodermis	CH	0.93	0.5	1.5	38.8	0.000	0.000	0.000	0.089	0.065
Shape of needle in cross-section [SW/ST]	NS	2.49	1.97	2.98	7.4	0.000	0.000	0.000	0.758	0.000
Shape of endodermis in cross-section [VCW/VCT]	VCS	1.58	1.09	1.91	5.1	0.396	0.727	0.379	0.212	0.491
Ratio of needle width/endodermis tube width [SW/VCW]	RW_1	3.92	3.19	6.00	7.3	0.000	0.000	0.002	0.000	0.000
Ratio of needle thickness/endodermis thickness [ST/VCT]	RW_2	2.50	2.04	3.00	5.9	0.040	0.736	0.000	0.005	0.000
Ratio of needle maximum width/needle width at 50% length [MW/W_50]	RW_3	1.05	0.91	1.15	3.0	0.330	0.900	0.081	0.471	0.031
Ratio of needle width at 95% length/needle width at 50% length [W_95/W_50]	RW_4	0.80	0.58	1.00	8.1	0.001	0.000	0.334	0.005	0.000
Location of the maximum width of the needle [BD/L*100%]	LMW	57.0	35.1	82.8	14.5	0.002	0.004	0.389	0.486	0.567
Ratio of number of stomata rows on adaxial to abaxial needle side (NRU/NRL)	RR	0.00	0.00	0.06		0.827	0.851	0.108	0.783	0.021
Ratio of number of stomata numbers on adaxial to abaxial needle side (NSU/NSL)	SR	0.00	0.00	0.00	0.0	0.998	0.997	0.931	0.997	0.016
Marcet coefficient [DV/SW × ST]	MC	16.06	1.87	44.26	27.3	0.000	0.000	0.000	0.000	0.009
Shape of epidermal cell in cross-section [EW/EH]	ES	1.10	0.78	1.67	10.0	0.457	0.004	0.010	0.002	0.000
Shape of hypodermal cell in cross-section [HW/HH]	HS	1.00	0.64	1.67	12.4	0.063	0.219	0.079	0.957	0.081
Shape of resin canal in cross-section [WC/HC]	CS	1.07	0.83	1.32	5.3	0.000	0.001	0.010	0.605	0.047

AET – *A. equi-trojani*, AA – *A. alba*, ABR – *A. × borisii-regis*, ANN – *A. nordmanniana*, ABR – *A. bornmuelleriana*, ACE – *A. cephalonica*, M – average, Min – minimal value, Max – maximal value, V – variation coefficient (only when lower than 50%)

* For NRU, NSU, NC, RR, SR, and CH.

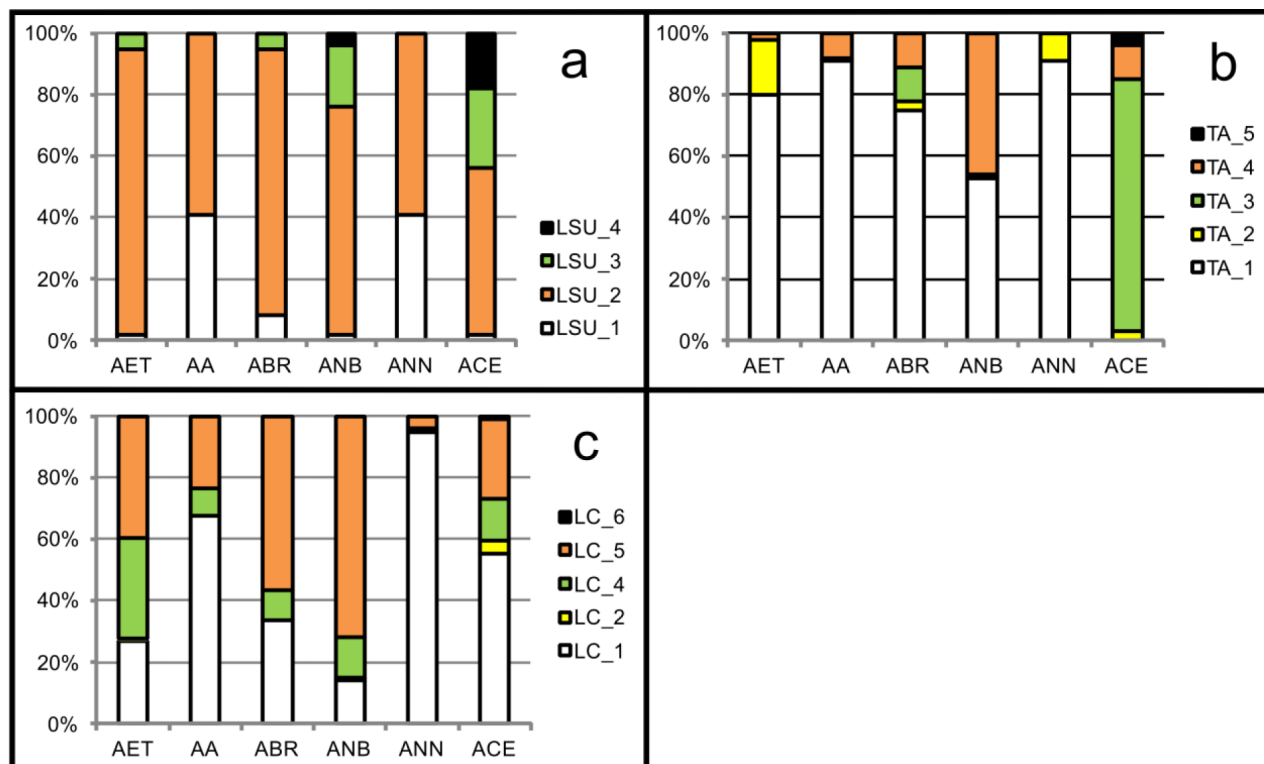


Figure 1. Percentages of needles in studied populations of *Abies equi-trojani* (AET), *A. alba* (AA), *A. ×borisii-regis* (ABR), *A. bornmuelleriana* (ANB), *A. nordmanniana* (ANN), and *A. cephalonica* (ACE) with: a- stomata on the adaxial side: lack of stomata (LSU_1), stomata on the apical part only (LSU_2), on the central and apical part (LSU_3), on the entire area of the needle adaxial side (LSU_4); b- different apex form: indented (TA_1), rounded (TA_2), obtuse (TA_3), obtuse-acute (TA_4), acute (TA_5), retuse (TA_6, not detected, omitted on the graph); c- position of resin canals: marginal lower (LC_1), marginal central (LC_2), marginal upper (LC_3, not detected, omitted on the graph), mesophyllum lower (LC_4), mesophyllum central (LC_5), mesophyllum upper (LC_6).

of agglomeration. A classification matrix was executed to find the percentage of individuals from each population and every taxon that could be properly classified into a particular group and to estimate the percentage of individuals that fell into another K-group (Sneath and Sokal, 1973). STATISTICA v. 9.0 software (StatSoft PL) was used for mathematical calculations.

3. Results

3.1. Character evaluation and correlation

From the set of characters, only MW, NRL, NSL, SW, ST, EW, EH, HW, HH, ES, and CS had normal distributions and homoscedastic variances. For comparisons of characters with a normal distribution but with inhomogeneous variances (A, P, L, W_95, W_50, BD, VCW, VCT, MT, DV, WC, HC, NS, VCS, RW_1, RW_2, RW_3, RW_4, LMW, MC, and HS) we used a t-test with Cochran–Cox adjustment (Stanisz, 2006).

The majority of needle characters in *A. equi-trojani*, 22 out of 39 analyzed, were variable at a relatively low level with a coefficient of variation (V) lower than 10% and as low as approximately 3% for RW_3 and NC. The highly

variable ($V \geq 25\%$) characters were A, DV, and CH. The extremely variable ones ($V \geq 100\%$) were NRU and RR.

The dimensional characters of the needles (A, P, and L), numbers of stomata (NSU and NRU), and set of character pairs on the cross-section of the needle (ST with VCW and VCT, HC with WC, and also MW with SW) were positively and significantly correlated with each other ($r \geq 0.90$). These relationships were detected for every taxon compared.

3.2. Differences between taxa

The average Kazdağı fir needle appeared to be approximately 24 mm long, shorter than that of *A. alba* but longer than that of *A. cephalonica*, and similar to those of *A. bornmuelleriana* and *A. nordmanniana*. The *A. equi-trojani* needle is relatively narrow and thick (MW = 1.76 mm, SW = 1.83 mm, and ST = 0.74 mm) when compared with those of other taxa. The position of the needle maximal width when compared to the needle length (LMW) appeared similar to that of *A. cephalonica* and significantly different from that observed in *A. alba* and *A. ×borisii-regis*. The needles of *A. equi-trojani* generally have no stomata in the central part of the adaxial side (NRU and NSU = 0 or close

to 0), but stomata were present at the apical parts in 93% of analyzed needles (Figure 1).

The majority *A. equi-trojani* individuals had, exclusively, needles with indented apices (TA). Only two of those analyzed individuals bore rounded needles, and a few had needles indented, obtuse-acute, and rounded (Figure 1). Considering the presence of stomata on the adaxial needle side (LSU) and the position of resin canals (LC), *A. equi-trojani* resembled *A. xborisii-regis* and *A. bornmuelleriana*, while it differed to a higher degree from *A. cephalonica* (Figure 1).

Pairwise comparisons between *A. equi-trojani* and other species using Student's t-test and/or the Mann-Whitney U test revealed that the highest number of characters differed significantly from *A. cephalonica* and *A. alba*, at 30 and 25, respectively, from 39 measured/estimated characters and proportions. The closest relationships to *A. equi-trojani* were revealed to be *A. bornmuelleriana* and *A. nordmanniana*, with 16 characters differing significantly in both cases (Table 2). From the measured character set and proportions, only the needle width (MW), the dimensions of resin canals on the needle cross-section (WC and HC), the ratio of needle width to epidermis width (SW/VCW), and the Marcet coefficient (MC) differed significantly between every possible pair of taxa. However, despite significant differences between mean values of characters for a particular taxon, the distribution ranges of every character overlapped substantially. Interestingly, the numbers of resin canals (NC), the ratio of VCW/VCT, and the shape of the hypodermal cell (HS) appeared almost the same in every species compared, while all the other characters differed significantly between *A. equi-trojani* and at least one of the compared taxa (Table 2).

The DA of the set of characters, except for NRU, NSU, RR, SR, and all variants of LSU, TA, and LC, revealed a central position of *A. equi-trojani*, most closely related to *A. xborisii-regis* and *A. bornmuelleriana* populations (Figure 2a). The populations of *A. xborisii-regis* are placed between *A. cephalonica* and *A. alba*, more closely to the latter species, with one population (ABR3) that could be included in the *A. alba* group (Figure 2b). The populations of *A. bornmuelleriana* and *A. nordmanniana* are generally closely related, and *A. equi-trojani* has been adjoined to this group (Figure 2b). The population has been differentiated mostly by RW_4, MT, MC, NS, and NRL, with partial Wilks' λ of 0.4816, 0.7507, 0.7771, 0.7869, and 0.7899, respectively. The two first discrimination variables are responsible for more than 70% of the total variation: U_1 for nearly 50% of the total variation, determined mostly by NS, RW_1, MC, and NRL, and U_2 for approximately 20% of the total variation, determined first of all by RW_4 and NS.

The DA of individuals allows us to distinguish fully between *A. alba* and *A. cephalonica*. The individuals of *A. equi-trojani*, *A. bornmuelleriana*, and *A. nordmanniana* formed one cloud (Figure 2c). Most of the individuals of *A. xborisii-regis* are closer to the group of *A. alba*, but some of them also entered the 95% confidence interval of *A. cephalonica* (Figure 2c). The individuals were differentiated to the highest degree by RW_4, MC, and NRL, with partial Wilks' λ of 0.5976, 0.8156, and 0.8805, respectively. The first two discrimination variables covered more than 87% of the total variation. U_1 , which covered more than 60% of the total variation, was determined mostly by RW_1, NS, and MC. The values of RW_1 and NS were significantly lower, whereas MC was higher in *A. cephalonica* when compared with all other taxa. U_2 , responsible for more than 23% of the total variation, was determined first by RW_4, NS, RW_1, and LMW.

Among compared taxa, the lowest values of the squared Mahalanobis distances were detected between *A. alba* and *A. xborisii-regis* (2.8), between *A. equi-trojani* and *A. bornmuelleriana* (4.4), and between *A. equi-trojani* and *A. xborisii-regis* (6.3). The highest distances were found between *A. alba* and *A. cephalonica* (29.3) and between *A. cephalonica* and *A. equi-trojani* (16.3). The classification matrix detected that the correct ranking of individuals to the species varied between 54% and 82%, with the highest number of incorrect classifications of individuals being those of *A. xborisii-regis* and *A. equi-trojani*. Fourteen individuals of the latter taxon were classified correctly, but seven were assigned to *A. bornmuelleriana*, three to *A. xborisii-regis*, and one to *A. alba*.

The agglomeration of the populations by shortest Euclidean distances per Ward's method revealed the closest connection of *A. equi-trojani* to *A. xborisii-regis* and to *A. alba* (Figure 3a). *A. alba* and *A. cephalonica* populations formed two separate groups (Figure 3b). Agglomeration by Mahalanobis distances gave a similar result (data not shown). The *K*-grouping detected three groups of populations as optimal. The first group formed populations of *A. cephalonica*; the second of *A. equi-trojani*, *A. bornmuelleriana*, and *A. nordmanniana*; and the third of *A. alba* and *A. xborisii-regis*. Groups of populations were differentiated mostly by NRL, NS, RW_1, RW_4, LMW, MC, HS, and CS. The average correctness of classification of individuals within populations to the group was above 60%, but it varied between about 30% in AA2 and more than 95% in ACE2. The geographical pattern of *K*-grouping indicated a connection of *A. equi-trojani* to *A. bornmuelleriana* and *A. nordmanniana*, and a distinction from *A. cephalonica* and *A. alba* (Figure 3c).

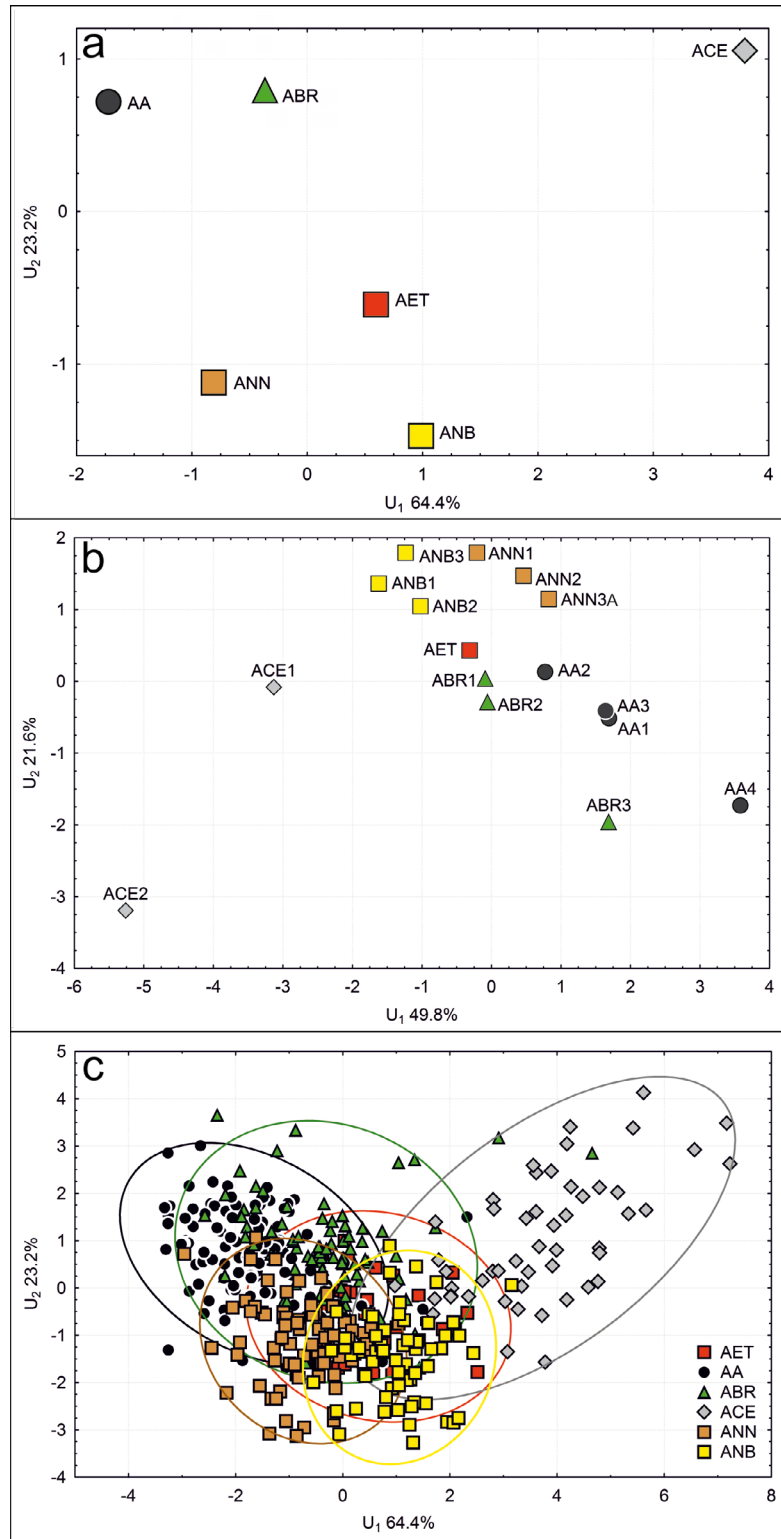


Figure 2. Results of discrimination analysis for *Abies equi-trojani* (AET), *A. alba* (AA), *A. ×borisii-regis* (ABR), *A. cephalonica* (ACE), *A. bornmuelleriana* (ANB), and *A. nordmanniana* (ANN) according to discrimination variables U_1 and U_2 : a- for species, b- for populations; c- for individuals (lines indicate 95% confidence intervals for each of the six taxa); acronyms of populations as in Table 1.

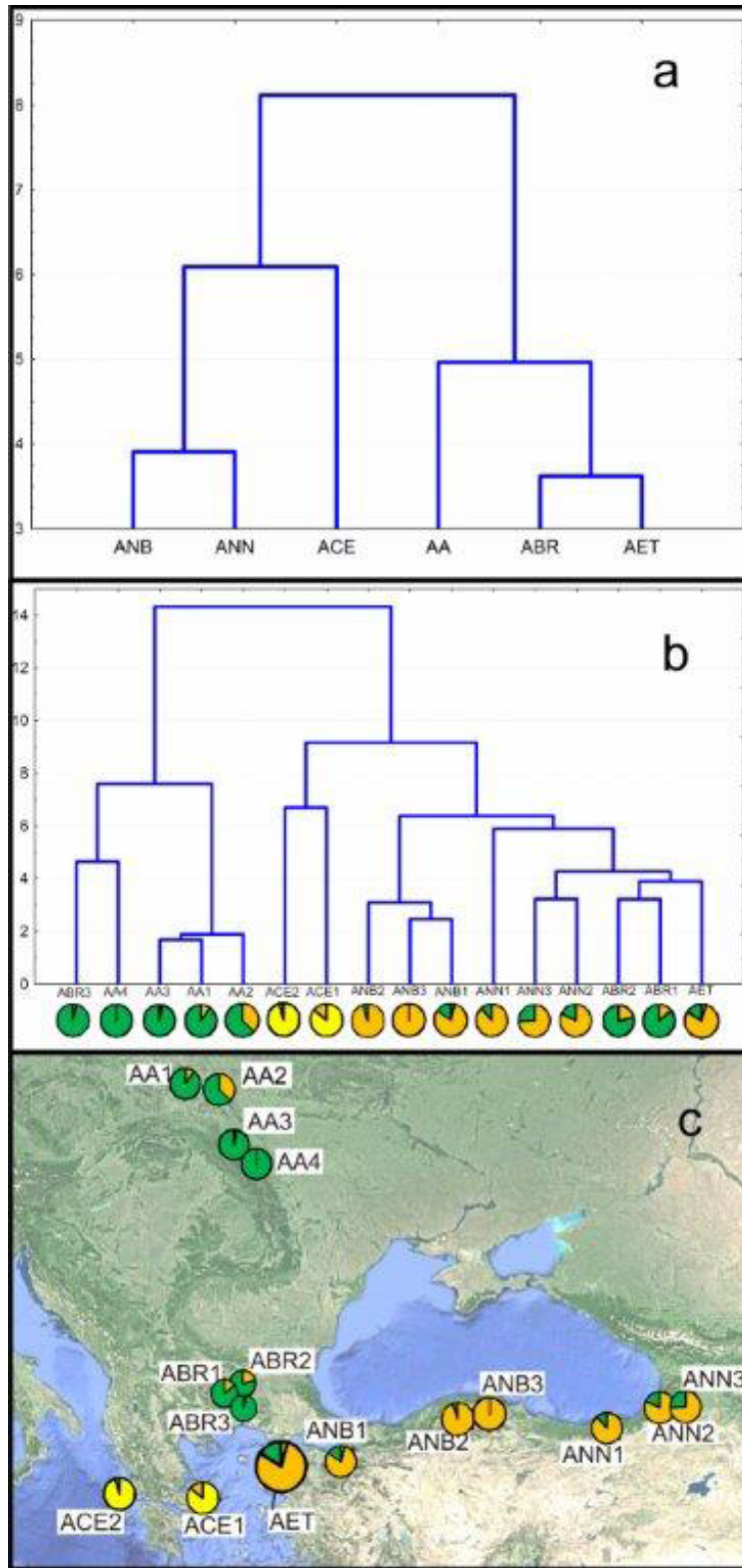


Figure 3. Agglomeration of populations of *Abies equi-trojani* (AET), *A. alba* (AA), *A. ×borisii-regis* (ABR), *A. cephalonica* (ACE), *A. bornmuelleriana* (ANB), and *A. nordmanniana* (ANN) using Euclidean distances from biometric data of species (a), populations (b), and geographical distribution of three groups of populations detected from *K*-means clustering (c); population acronyms as in Table 1.

4. Discussion

Several needle characters were used in the comparison of fir from Kazdađı with *A. cephalonica*, *A. alba*, and *A. ×borisii-regis* (Mitsopoulos and Panetsos, 1987); *A. cilicica*, *A. nordmanniana*, and *A. bornmuelleriana* (Bagci and Babaç, 2003); and *A. bornmuelleriana* (Kurt et al., 2016). Mitsopoulos and Panetsos (1987) used needles from the seedlings of compared taxa growing in common garden conditions. Two subsequent studies were based on needles sampled from adult trees in natural populations, but from different positions on the crowns (Bagci and Babaç, 2003) or from upper crown parts of selected trees of *A. bornmuelleriana* (Kurt et al., 2016). The methods of sampling probably explain the differences between the results obtained in these studies and our data (Table 3), which were obtained from needles collected from the southern expositions of lower parts of the crowns of adult, cone-bearing trees. The known data from the most detailed study of the needle characteristics by Bagci and Babaç (2003) concerned mostly the needle length (L),

width (MW), apex form (TA), and position of the resin canals (LC). Apart from those mentioned, we examined several characters that have not been analyzed until now. The detected average values of most characters have been specified for the first time on the representative material from the natural locality of *A. equi-trojani* in Kazdađı (Figure 1).

It is generally accepted that the characteristics of the cones (shape of cone scales, shape of bracts, and level of their exertion beyond the cone), the shape of buds, arrangement of the needles, and forms of their apices are the most important characteristics necessary to distinguish fir species (Mattfeld, 1925; Liu, 1971; Bagci and Babaç, 2003). *Abies equi-trojani* differs from the most closely related *A. bornmuelleriana* and *A. nordmanniana* by having glabrous young shoots, usually nonresinous buds, and frequently acute needle apices (Coode and Cullen, 1965; Cullen and Coode, 1965). We detected a statistically significant difference ($P \leq 0.01$) between *A. equi-trojani* and all the other studied species in needle

Table 3. Data values of *Abies equi-trojani* needle characters known from the literature; the values obtained in our study are bolded (with minimum–maximum); particular authors’ data are in the form of mathematical mean (M) ± standard deviation (SD) or ± standard error (SE), as indicated in the column ‘Remarks’.

Character	Value	Source of data	Remarks
L [mm]	24.03 ± 3.84	Mitsopoulos and Panetsos, 1987	Needles from seedlings planted in Greece (M ± SD)
	15–25	Schütt, 1991	Undetermined needle position
	18.9 ± 0.9	Kurt et al., 2016	Needles from upper part of tree crown, Kazdađı (M ± SE)
	18.1 ± 0.6	Kurt et al., 2016	Needles from upper part of tree crown, average (M ± SE)
	26.3 ± 0.37	Bagci and Babaç, 2003	Needles from three different positions of tree crowns from Turkey (M ± SE)
	25–30	Gaussen, 1928	Sterile branches
	23.79 (12.9–40.5)		
MW/SW [mm]	1.76 ± 0.14	Mitsopoulos and Panetsos, 1987	Needles from seedlings planted in Greece (M ± SD)
	1.5	Kurt et al., 2016	Needles from upper part of tree crown, Kazdađı
	1.4	Kurt et al., 2016	Needles from upper part of tree crown, average
	1.90 ± 0.02	Bagci and Babaç, 2003	Needles from three different positions of tree crowns from Turkey (M ± SE)
	1.5–2	Gaussen, 1928	Sterile branches
	1.76 (1.23–2.04)		
ST [µm]	590 ± 0.04	Mitsopoulos and Panetsos, 1987	Needles from seedlings planted in Greece (M ± SD)
	511 ± 20.2	Bagci and Babaç, 2003	Needles from three different positions of tree crowns from Turkey (M ± SE)
	739.2 (559.9–973.1)		
(WC+HC)/2 [µm]	50.5 ± 5.21	Bagci and Babaç, 2003	Needles from three different positions of tree crowns from Turkey (M ± SE)
	71.65 (34.24–126.26)		

width (MW), width and height of resin canals (WC, HC), the Marcet coefficient (MC), and the ratio of needle to endodermis tube width (RW_1). The needles of *A. equi-trojani* were the narrowest and had the smallest resin canals of all compared taxa, which is congruent with data from Bağcı and Babaç (2003), who compared Anatolian firs. Additionally, we detected several characters differing at a significant level between *A. equi-trojani* and other taxa (Table 2). It should be stressed, however, that none of them allows us to distinguish between compared taxa unequivocally. For this reason, a set of data must be used.

Multivariate analyses allowed us to distinguish the species and the populations representing every one of them (Figures 2a and 3a and Figures 2b and 3b, respectively). On the level of individual trees, *A. alba* and *A. cephalonica* can be distinguished with a very high probability (about 90%), but individuals of the other taxa formed a group placed between *A. alba* and *A. cephalonica* (Figure 2c). *Abies ×borisii-regis* from the Pirin and Rila mountains in Bulgaria appeared closer to *A. alba*, which could be interpreted as a confirmation of the hybrid origin of this taxon, with a more prominent influence of *A. alba* in the northernmost Bulgarian populations (compare maps 152 and 154 in Jalas and Suominen, 1973; Panetsos, 1975; Mitsopoulos and Panetsos, 1987; Bella et al., 2015; Krajmerová et al., 2016). *Abies ×borisii-regis* is variable and its local populations could present different types of morphology (Panetsos, 1975; Mitsopoulos and Panetsos, 1987; Christensen, 1997). Differences between the population of ABR3 and two other populations of *A. ×borisii-regis* could result from different influences of the parental *A. alba* and *A. cephalonica*, as the king Boris fir was found as nonmonophyletic (Bella et al., 2015; Krajmerová et al., 2016). Differences between populations could also reflect an impact of slightly different site conditions (Hultine and Marshall, 2000; Tiwari et al., 2013; Donnelly et al., 2016); however, this hypothesis should be verified in detail ecological studies.

Abies equi-trojani individuals are dispersed among individuals of *A. bornmuelleriana* and *A. nordmanniana*, forming one group. It should be noted, however, that the better part of *A. nordmanniana* individuals are more like *A. alba*, while *A. bornmuelleriana* and *A. equi-trojani* more closely resemble *A. cephalonica* (Figure 2c). This could be interpreted as confirmation of the hypothesis of a common ancestral taxon of the latter three species (Linares, 2011) or a hybrid origin of *A. equi-trojani* and *A. bornmuelleriana* that could have resulted from cross-pollination between *A. cephalonica* and *A. nordmanniana* (Klaehn and Winieski, 1962; Ata and Korgavus, 2012; Kormutak et al., 2013). The intermediate position of *A. equi-trojani* between *A. cephalonica* and *A. bornmuelleriana* was also detected in the wood anatomy (Aytug, PhD thesis, 1958) and

pollen characters (Aytug, 1959). Ancient hybridization between northern Anatolian populations of firs has been confirmed lately by the discovery of *A. ×olcayana*, which is intermediate between *A. equi-trojani* and *A. bornmuelleriana* (Ata and Merev, 1981, 1987).

Genetic investigations of the Kazdağı fir indicated a relatively high level of diversity within but a rather low differentiation between four known populations of the species (Velioğlu et al., 1998). Comparative genetic studies placed *A. equi-trojani* close to *A. cephalonica*, *A. nordmanniana*, and *A. bornmuelleriana* using RLFP haplotypes, cpDNA markers, and mtDNA haplotypes (Liepelt et al., 2010); close to *A. ×borisii-regis* in isozyme marker analysis (Scaltsoyiannes et al., 1999); and very close to *A. bornmuelleriana* and/or *A. nordmanniana* using cpDNA markers (Kaya et al., 2008). *Abies equi-trojani* has the same mitochondrial DNA haplotype as found in *A. cephalonica*, *A. bornmuelleriana*, and *A. nordmanniana* (Ziegenhagen et al. 2005; Liepelt et al., 2010), also among individuals planted in the botanic gardens in Central Europe (Pawlaczyk et al., 2015). These close relations between *A. equi-trojani* and other fir taxa from southeastern Europe and northern Anatolia have also been confirmed in our biometric study on needle morphological and anatomical characteristics.

The similarities of *A. equi-trojani* to *A. bornmuelleriana* and *A. nordmanniana* in the needle characteristics and close relationships between these three taxa detected in our investigation seem to support their infraspecific systematic position proposed by Cullen and Coode (1965). The analysis of fir speciation within the Mediterranean indicated the historical development of the Balkan and North Anatolian contemporary species from one Tertiary ancestor (Liepelt et al., 2009, 2010). Its hypothetically broad geographic range was divided since the Miocene into the Balkan and Middle Asiatic regions, then isolated during Miocene and Pliocene geological and climatic events, which caused the formation of the current taxa (Linares, 2011). Possible contact and gene exchange during the Pliocene and Pleistocene climate oscillations were likely responsible for the observed morphological and genetic differentiation of the Balkan and Anatolian taxa (Scaltsoyiannes et al., 1999; Kaya et al., 2008; Liepelt et al., 2010; Linares, 2011), as firs retain a high level of ability to cross-pollinate among species (Kormutak et al., 2013; Bella et al., 2015; Krajmerová et al., 2016). These historical events were the reason for the muted morphological differences between *A. equi-trojani*, *A. bornmuelleriana*, and *A. nordmanniana*, independently of the systematic status assigned to each of them. The same history explained the greater differences between the three taxa mentioned above when compared with *A. alba* and *A. cephalonica*. The similarity of *A. equi-trojani* and also *A. bornmuelleriana* and *A. nordmanniana*

to *A. ×borisii-regis* could confirm a common origin for all these taxa, the latter, however, being an effect of ongoing hybridization between *A. alba* and *A. cephalonica* (Bella et al., 2015; Kajmerová et al., 2016).

Consequently, in the morphological and anatomical characters of their needles, *A. equi-trojani* is close to *A. bornmuelleriana*, *A. nordmanniana*, and *A. ×borisii-regis*. Nonetheless, a set of characters allows us to distinguish the population of *A. equi-trojani* from populations of *A. alba*, *A. bornmuelleriana*, *A. nordmanniana*, *A. ×borisii-regis*, and *A. cephalonica*. Likewise, multivariate analyses distinguish *A. equi-trojani* from all other species, but only on the population level. Individuals of *A. equi-trojani* could be like those of *A. bornmuelleriana*, *A. nordmanniana*,

or *A. ×borisii-regis* but not like those of *A. alba* or *A. cephalonica*. It should be noted, however, that we studied only one Kazdağı fir population, covering a relatively small part of the species geographic range. Therefore, further comparative studies should be carried out on other populations of the species, including the newly described *A. ×olcayana*.

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