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# Diversity in needle morphology and genetic markers in a marginal *Abies cephalonica* (Pinaceae) population

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**Abstract.** Differences in needle traits of coniferous tree species are considered as the combined result of direct environmental pressure and specific genetic adaptations. In this study, diversity and differentiation within and among four *Abies cephalonica* subpopulations of a marginal population on Mt. Parnitha - Greece, were estimated using needle morphological traits and gene markers. We tested the connection of morphological variability patterns of light and shade needles with possible adaptation strategies and genetic diversity. Six morphological characteristics were used for the description of both light and shade needles at 100 trees, describing needle size and shape, stomatal density and needle position on the twigs. Additionally, six RAPD and three ISSR markers were applied on DNA from the same trees. Light needles were significantly different than shade needles, in all traits measured, apparently following a different light harvesting strategy. All four subpopulations exhibited high genetic diversity and the differentiation among them was relatively low. Differences among populations in light needles seemed to depend on light exposure and aspect. In shade needles, the four subpopulations seemed to deviate stronger from each other and express a rather geographic pattern, similarly to the genetic markers. Two of the subpopulations studied were lost during a wildfire, two years after sampling. Although the subpopulations burnt were most diverse and most differentiated, we expect a large part of the total genetic diversity of the burnt trees to still exist in the surviving subpopulations, since gene flow must have been effective in keeping all subpopulations connected.
**Keywords** *Abies cephalonica*, conifer, needle morphology, genetic diversity, adaptation.

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## Introduction

Survival of woody plants depends on the development of morphological traits adapted to multiple and complex aspects of environmental pressure. Along an altitudinal gradient and topography, crucial environmental factors such as temperature, light and moisture change drastically, even in very short distances (Schulze et al. 2002). In particular, conifers usually inhabit places where non optimal environmental conditions prevail. In Mediterranean countries, coniferous species may well grow and form extended forests in harsh environments that often occur at upper montane levels towards the treeline.

In order to adapt, conifers have developed needle shaped leaves of small size, surrounded by thick waxy layers (Millar 1974). This type of leaves protects them from intense UV radiation and heat losses during cold nights, as well as enables them to maintain sufficient photosynthetic activity under low temperatures. Conifer needles are acclimated to their environment and develop adaptive phenotypic responses by altering their length, width, number of stomata, as well as their angle towards the shoot or by forming needle clumping (Niinemets et al. 2002, Smolander & Stenberg 2003, Niinemets 2010, Gebauer et al. 2011). Those traits change differently from the base to the top of the tree and especially from the outside (light needles) to the interior of the crown (shade needles), mainly due to light availability. Light is a common stress factor, not only in terms of quantity but also of quality, in the high altitudes where conifers are often present. As the altitude increases, solar radiation becomes more intense and many times causes photoinhibition in photosynthesis (Werner et al. 2001). To overcome that stress, conifers create three-dimensional shoots with needles in clumping. Additionally, light needles present higher thickness, higher leaf mass per area, higher width or length and usually higher stomata number on their upper surface (Ishii et al. 2012, Sprugel et al. 1996, Niinemets 1997, Stenberg et al. 1999, Youngblood & Ferguson 2003). Those traits follow a different pattern in the interior of the canopy, where the light reaches the needles by diffusion. Shade needles exhibit lower thickness, reduced width and length, are widely parted on shoot and no mutual shading exists. They further present lower stomatal density and more uniform allocation of chlorophyll per leaf area (Abrams & Kubiske 1990, Mori & Takeda 2004, Sancho-Knapik et al. 2014). These morphological differences in needle size, shape and position depict two different strategies towards solar radiation. In shoots exposed to the sun, incoming radiation is spread out more efficiently over a large number of thick needles at many possible angles, minimizing losses due to saturation (Carter & Smith 1985, Oker-Blom et al. 1991). In shoots of the inner canopy, a single horizontal layer of thinner needles is set to receive radiation, minimizing the cost in leaf construction and maintenance for the whole tree (Leverenz & Hinckley 1990). Both types of needles contribute in common to the total tree performance and therefore both needle types must be considered in morphological studies of conifers (Gebauer et al. 2011).

Needle phenotypes are used in studies where different tree populations are compared and they are considered as the combined outcome of direct environmental pressure and the expression of specific genetic adaptations (see also Tsumura et al. 2014). Several authors report of different levels of genetic contribution to the expression of these traits. As an example, Xing et al. (2014) report that 10 out of 24 needle morphological traits were found to be under genetic control among full sib families in an artificial hybridization experiment of *Pinus densata*. Differences in needle traits have been described as the outcome of differentiated adaptation strategies between coniferous species (i.e. Grassi & Giannini 2005) and among populations of the same species (i.e. Mitsopoulos & Panetsos 1987, Wright et al. 1998).

The Greek fir, *Abies cephalonica* Loudon, is one of the three native fir taxa that occur in Greece and the only endemic. It is the dominant forest tree, in pure and mixed stands, in the mountains of the central and southern part of the country (Mattfeld 1927, Mitsopoulos & Panetsos 1987, Farjon 2010). Its distribution can be regarded as island-type, since there is no physical connection among most of the *A. cephalonica* populations (Mitsopoulos & Panetsos 1987, Christensen 1997), while some of these populations have been reduced and are currently occupying a restricted area only. All the *Abies* species around the Meditteranean basin and Black sea have originated from a common Tertiary ancestor that gave rise to the current fir taxa during Pliocene, Pleistocene and Holocene (Scaltsoyiannes et al. 1999, Linares 2011). *A. cephalonica* originated after the Pliocene and following its subsequent contact with *A. alba* during the ice ages, the hybrid *A. ×borisii-regis* derived (Linares 2011), which is combining morphological characters of the two species (Christensen 1997). The hybrid origin of *A. ×borisii-regis* has been supported by various studies employing morphological, biochemical and molecular traits (e.g. Mattfeld 1930, Mitsopoulos & Panetsos 1987, Fady et al. 1992, Drouzas 2000, Bella et al. 2015). An alternative hypothesis, that *A. ×borisii-regis* represents the *Abies* ancestor was rejected by Mattfeld (1930) but was put forward by Turril (1937) and supported by Scaltsoyiannes et al. (1999). A recent study employing maternally and biparentally inherited molecular markers suggested that *A. ×borisii-regis* is the product of recent introgression between *A. cephalonica* and *A.* alba (Krajmerova et al. 2015). From Central Greece northwards, Greek fir is gradually replaced by *A. ×borisii-regis* and mixed forests of the two taxa exist (Mitsopoulos & Panetsos 1987, Fady & Conkle 1993). Individual trees with characters of *A. cephalonica* occur mixed with intermediate forms well into northern Greece (Farjon 2010).

*A. cephalonica* is a limestone tolerant that may develop on shallow or rocky substrate, while populations closer related to *A. ×borisii-regis* are quite demanding on site quality and can be found regularly on deep soils (Barbéro & Quézel 1976, Schütt 1994). Greek fir populations grow on altitudes mainly between 600-2100 m, but the optimum altitudinal range is considered to be from 800 to 1200 m (Panetsos 1975). This zone receives the largest part of the annual precipitation during autumn and winter and may suffer from summer drought (Aussenac 2002, Gouvas & Sakellariou 2011). Drought can become severe on shallow soils with reduced waterholding capacity, as soil moisture is considered to be the crucial limiting factor for growth and survival of the Greek fir (Schütt 1994). *A. cephalonica* has the ability to withstand dry habitat conditions in comparison to other Mediterranean firs (Aussenac 2002, Bergmeier 2002). However, a decline and various damages, including crown dieback, needle loss, needle discoloration and death of twigs, branches or death of the whole tree have been reported for *A. cephalonica*, which are attributed to drought, climatic change, air pollution and pathogens or insects (Raftoyannis et al. 2008). Extensive dieback of Greek fir trees and forest decline has shown to be caused by prolonged periods of drought (Athanassaki 1999) or air pollution (Heliotis et al. 1988).

Several studies have compared *A. cephalonica* populations growing naturally or planted in common garden experiments in Greece and in other European countries. Considerable variation, both among and within populations has been found in morphological – anatomical traits (Mattfeld 1927, Mattfeld 1930, Panetsos 1975, Mitsopoulos & Panetsos 1987, Fady et al. 1991), terpenes (Mitsopoulos & Panetsos 1987, Fady et al. 1992), biochemical and molecular markers (Fady & Conkle 1993, Kormutak et al. 1993, Scaltsoyiannes et al. 1999, Drouzas 2000, Parducci et al. 2001, Bella et al. 2015, Krajmerova et al. 2015). Some morphological traits, such as needle size, needle sharpness and the number of stomata on the upper side of the needle seem to change gradually due to adaptation to ecological clines related to drought tolerance (Mitsopoulos & Panetsos 1987, Fady et al. 1991).

Mt. Parnitha (also known as Mt. Parnes) is located on the north side of the broader Athens region and borders with urban areas from all sides (Fig. 1). Covering an area of about 30,000 ha, it has 16 peaks higher than 1000m, with "Karavola" being the highest (1413 m). The climate of Mt. Parnitha area is characterized by high summer temperatures and drought, while the mountain and especially its southern slopes are exposed to the influence of factors caused by the close proximity to the urban area of Athens, such as increased solar radiation and pollution (Heliotis et al. 1988). Even though human pressure must have been always very high in Mt. Parnitha, given its close proximity to Athens, this mountain is characterized by high species biodiversity and a rich mosaic of numerous ecosystems (Aplada et al. 2007). Among them, the fir forest on the higher altitudes covers 3,500 ha and is protected since 1961. The fir on Mt. Parnitha is a restricted and isolated *A. cephalonica* population that survives in adverse environmental conditions compared to the other fir forests in Greece. In addition, damages caused by biotic or abiotic factors and lack of regeneration have been reported (Tsopelas et al. 2004, Apatsidis et al. 2005, Aplada et al. 2007), especially on the southern slopes of the mountain. Based on isozyme analyses, the fir population from Mt. Parnitha showed similarities with other isolated Greek fir populations (e.g. from Cephalonia island, Mt. Elikonas and Evia island), a group that was differentiated from the populations of the core distribution of *A. cephalonica* (and even *A.* ×*borisii-regis*) in Greece (Drouzas 2000). Similarity between the fir populations of Mt. Parnitha and Evia island has been also shown by terpene analysis (Fady et al. 1992). Therefore, the fir forest of Parnitha may be considered to be an ecotype, genetically differentiated and adapted to adverse and unfavorable conditions. In 2007, two years after sampling, a wildfire destroyed the largest part of the fir forest (69.5%), an area of 2,049 ha (Figure 1). Only the northern and easternmost parts of the forest survived. Since fir is unable to regenerate naturally after fire, reforestation activities have been planned and partially implemented by the competent authorities (Ganatsas et al. 2012).

In this study, we will attempt to describe and explain the patterns of diversity and differentiation within and among four *A. cephalonica* subpopulations of Mt. Parnitha in needle morphology and gene markers and to seek for relations between them. We will more specifically try to answer the following questions: (i) Are there any differences in morphological traits between light and shade needles and are these differences consistent over different subpopulations? (ii) How is diversity in morphological traits of light and shade needles and gene markers distributed within and among subpopulations and what are the possible reasons for this distribution? Having used material from (now) burnt and remaining parts of the fir forest, we will further try to answer questions regarding the loss of genetic diversity, due to the fire and the suitability of the remaining forest to provide seed for restoration of the initial fir population.

## Materials and methods

Fir needles were collected in 2005 on Mt. Parnitha, two years before the fire that destroyed the largest part of the forest. The needles were collected from 100 trees growing in four different subpopulations, "Ag.Petros" on the north-facing slope, "Flaburi" on the south-eastern slope, "Kyra" and "Ag.Triada" on the south slope of the mountain (Figure 1). In each subpopulation 25 trees were sampled. The fire of 2007 destroyed subpopulations Kyra and Ag.Triada. From each tree, two branches were collected, one from the outer side of the crown and one from the inner side. We considered the

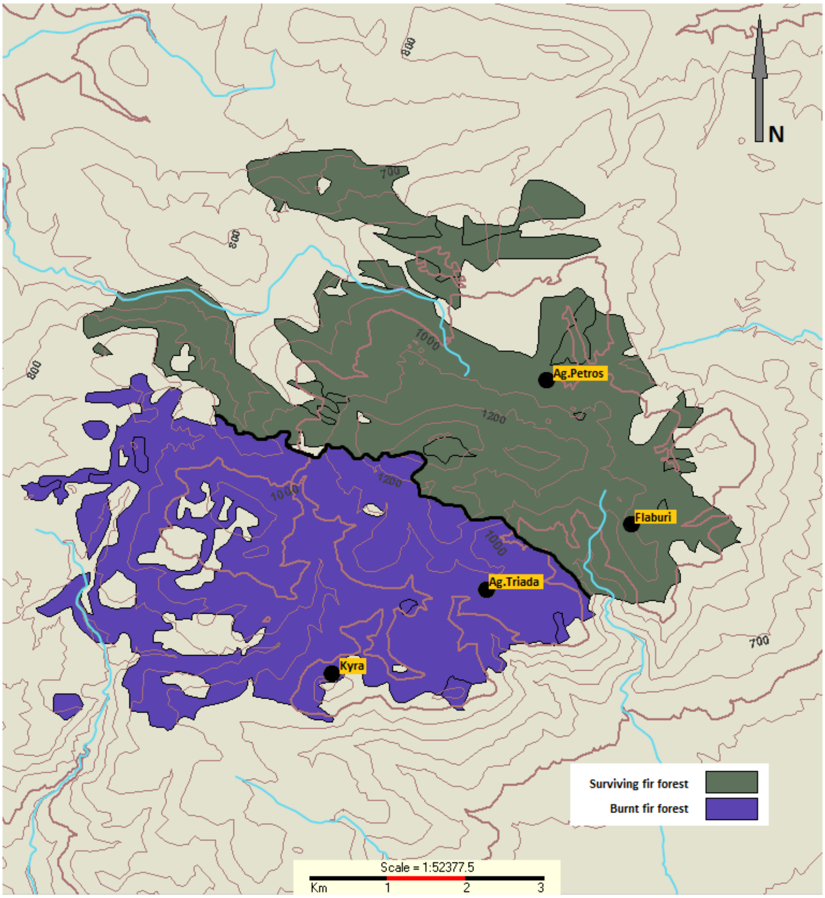

**Figure 1** Map of the fir forest on Mt. Parnitha and the four subpopulations sampled

needles of the outer branches as light needles and the ones from the inner side of the crown as shade needles. The needles were dried using silica gel and stored.

Six morphological characteristics were used for the description of both light and shade needles. These characteristics have been described as adaptive in studies where Greek fir species and populations were compared (Mitsopoulos & Panetsos 1987, Fady et al. 1991). Three of these traits were measured on 20 needles from each tree (10 shade and 10 light needles), in cm: needle length (Nlenght), maximum needle width (Nwidth) and needle sharpness (Sharpness; as the distance between the top of the needle and the point on the primary axis where the width of the needle becomes one mm). Three additional traits were recorded for each tree and were classified in categories. The clumping level of the needles on one light shoot and one shade shoot for each tree (Clumping) was given a score from one (pectinate form) to four (radially spreading outwards from all around the shoot, or "brush-like" form). The angle of the needles on one light twig and one shade twig for each tree (Nangle) was given a score from one (straight angle) to three (acute angle). Finally, on each needle, the quantity of the stomata on the upper surface of the needles (Stomata) was described in five classes, from one (very few) to five (abundant). Average values for all traits were estimated for each tree, for each subpopulation and for each class of light exposure. Means were compared using one and two-way ANOVA and correlations between variables were described using the linear regression model with R packages Rcmdr (Fox 2005) and ggplot2 (Wickham 2009). A principal component analysis (PCA) and a multiple factor analysis (MFA) were used to describe relationships among variables, groups of variables, trees, subpopulations and light exposure classes, using FactoMineR (Lê et al. 2008). In this package, cross validation approximation (GCV) was used to calculate the best number of PCA dimensions (Josse & Husson 2012). A Markov Chain Monte-Carlo simulation (MCMC) model was employed to classify each tree in morphological clusters for light and shade needles and to calculate the probability of each tree to belong to each cluster, using Geneland (Guillot et al. 2012). The simulation with the best mean posterior density for all clusters was chosen after ten individual runs of the non-spatial model.

DNA was extracted from dry needles using the DNeasy Plant Kit (Qiagen) following manufacturer's instructions. The quality and quantity of the extracted DNA was tested on 0.8% agarose gels after staining with ethidium bromide. Six RAPD and three ISSR primers were employed (Table 1).

For both RAPD and ISSR, the PCR amplifications were performed in a total volume of 15 µl containing 50ng DNA template, 2 mM MgCl2, 0.1 mMdNTPs, 0.3 µM primer, Q solution and 1U HotStartTaq Polymerase (QIAGEN®). The amplification conditions for RAPD were 94 °C for 15 min, followed by 45 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The same amplification conditions were used for ISSR, except for the annealing temperature which was set to 52°C. All the PCR reactions were carried out in an Applied Biosystems® Veriti® 96 well thermal cycler. The PCR products were visualized through electrophoresis on 1.2% agarose gels after staining with ethidium bromide. PCR amplifications were repeated twice and only the reproducible bands were scored as present (1) or absent (0) in each zone and a binary data matrix was prepared. Both RAPD and ISSR are dominant and highly polymorphic markers, particularly useful in studying the genetic variation within populations. Given their similar properties, the results from RAPD were merged with those from ISSR and the subsequent analyzes were done in the merged data-set.

Assuming HW equilibrium, allele frequencies were calculated using the square root method. Based on these frequencies, pairwise individual comparisons were used to describe the partition of genetic diversity within and among subpopulations with a hierarchical analysis of molecular variance (AMOVA) and 999 permutations, using Genalex 6.5 (Peakall & Smouse 2006, 2012). The AMOVA followed the approach for dominant markers of Huff et al. (1993) and Peakall et al. (1995). The

**Table 1** Sequences of six RAPD and three ISSR primers used

| Primer | Sequence |
|---|---|
| RAPD | |
| OPX06 | 5'-ACGCCAGAGG-3' |
| OPZ06 | 5'-GTGCCGTTCA-3' |
| OPZ13 | 5'-GACTAAGCCC-3' |
| OPZ19 | 5'-GTGCGAGCAA-3' |
| OPZ20 | 5'-ACTTTGGCGG-3' |
| OPC16 | 5'-CACACTCCAG-3' |
| ISSR | |
| M809 | 5'-AGAGAGAGAGAGAGAGG-3' |
| M814 | 5'-CTCTCTCTCTCTCTCTA-3' |
| M822 | 5'-TCTCTCTCTCTCTCTCA-3' |

percentage of polymorphic zones (P%), the effective number of alleles (Ne) and the average gene diversity (He) (Nei 1973) were used to describe the diversity within subpopulations. The unbiased genetic distances among subpopulations (Nei 1987) were used to describe differentiation and produced a UPGMA dendrogram visualized with TreeView (Page 1996). The R package adegenet (Jombart 2008) was used to perform a PCA and a discriminant analysis of principle components (DAPC) in order to cluster the individual trees and to compare cluster membership of trees among subpopulations (Jombart et al. 2010). A MCMC simulation was used to infer the number of panmictic groups and to describe the genetic structures of subpopulations according to the classification probability of each tree in these groups, with Geneland (Guillot & Santos 2010) and by keeping the simulation with the best mean posterior density after ten runs.

For the pairwise comparison of the morphological and the genetic dissimilarity tables among individuals, a Mantel test (Mantel 1967) was performed with the zt software (Bonnet & van de Peer 2002).

## Results

Mean values of needle width (Nwidth) and needle angle (Nangle) were significantly different among subpopulations for needles exposed to light. Light needles were thicker and their average angle on the twig was less obtuse in Ag.Petros (Table 2). The other four variables

**Table 2** Means and Analysis of Variance (ANOVA) of needle traits for four subpopulations, two levels of shading and the subpopulation x shading interaction

| Light needles | | Nlength | Nwidth | Sharpness | Clumping | Nangle | Stomata |
|---|---|---|---|---|---|---|---|
| Ag.Petros | mean | 2.25 | 2.08 | 0.81 | 3.92 | 1.64 | 2.70 |
| | (SD) | (0.28) | (0.15) | (0.28) | (0.28) | (0.70) | (0.87) |
| Ag.Triada | mean | 2.09 | 2.04 | 0.69 | 3.76 | 1.16 | 2.68 |
| | (SD) | (0.21) | (0.08) | (0.25) | (0.52) | (0.37) | (1.03) |
| Flaburi | mean | 2.10 | 2.00 | 0.74 | 3.96 | 1.20 | 2.74 |
| | (SD) | (0.29) | (0.11) | (0.27) | (0.20) | (0.41) | (0.91) |
| Kyra | mean | 2.19 | 2.03 | 0.75 | 3.92 | 1.16 | 2.92 |
| | (SD) | (0.30) | (0.07) | (0.24) | (0.28) | (0.37) | (0.81) |
| ANOVA sig. | | ns | * | ns | ns | * * | ns |
| Shade needles | | Nlength | Nwidth | Sharpness | Clumping | Nangle | Stomata |
| Ag.Petros | mean | 2.03 | 1.77 | 1.09 | 2.76 | 1.88 | 1.15 |
| | (SD) | (0.31) | (0.18) | (0.32) | (0.66) | (0.33) | (0.22) |
| Ag.Triada | mean | 2.06 | 1.87 | 0.86 | 2.48 | 1.72 | 1.38 |
| | (SD) | (0.20) | (0.14) | (0.20) | (0.77) | (0.46) | (0.53) |
| Flaburi | mean | 1.98 | 1.75 | 0.92 | 2.88 | 1.80 | 1.26 |
| | (SD) | (0.27) | (0.16) | (0.28) | (0.78) | (0.50) | (0.40) |
| Kyra | mean | 2.10 | 1.89 | 0.80 | 2.96 | 1.84 | 1.62 |
| | (SD) | (0.29) | (0.11) | (0.21) | (0.54) | (0.37) | (0.57) |
| ANOVA sig. | | ns | * * | * * | ns | ns | * * |
| All subpopulations | | Nlength | Nwidth | Sharpness | Clumping | Nangle | Stomata |
| Light | mean | 2.16 | 2.04 | 0.75 | 3.89 | 1.29 | 2.76 |
| | (SD) | (0.28) | (0.11) | (0.26) | (0.35) | (0.52) | (0.90) |
| Shade | mean | 2.04 | 1.82 | 0.92 | 2.77 | 1.81 | 1.35 |
| | (SD) | (0.27) | (0.16) | (0.28) | (0.71) | (0.42) | (0.48) |
| ANOVA sig. | | * * | * * * | * * * | * * * | * * * | * * * |
| Subpop. X shading | | Nlength | Nwidth | Sharpness | Clumping | Nangle | Stomata |
| ANOVA sig | | ns | * * | ns | ns | ns | ns |

Note. Abbreviations: SD - standard deviation, ns - non significant, * - significant at 5%, ** - significant at 1% and *** - significant at 1‰.

did not show significant differences among the subpopulations for light needles. For shade needles, three variables had significantly different mean values among subpopulations. Needles were thicker and demonstrated a larger stomatal density in Kyra and then in Ag.Triada, while they were significantly sharper in Ag.Petros (Table 2). Differences of the mean values of all traits between different shading classes were significant. Light needles were longer, thicker, less sharp and with a higher stomatal density than shade needles. The angle between the light needles was less obtuse and clumping level was towards "brush-like" when branches and needles were exposed to light. Shoots with shade needles showed a more pectinate structure. A significant interaction between subpopulations and shading class was found only for needle width (Table 2).

Trends in differences among populations between light and shade needles were different for most traits. Light needles were longer and thicker in Ag.Petros, while shade needles were longer and thicker in Kyra. Regarding the angle of the needles, Ag.Petros had the least obtuse angle when light needles were viewed, while there were no obvious differences in this trait when shade needles were considered. For the rest of the traits, the differences among subpopulations followed the same trend for light and shade needles. However in needle clumping and sharpness, subpopulation Kyra showed a different pattern for different shading classes.

A PCA was performed for both light and shade needles. The generalized cross validation method (GCV) showed that traits describing the light needles did not form any significant principal component, while the same traits for shade needles formed only one principal dimension. In both cases, the three first components explained a similar percentage of the total diversity (64.61% for light needles and 66.7% for shade needles). However, only the shade needles showed a significant difference in eigenvalue scaling and a first PCA axis

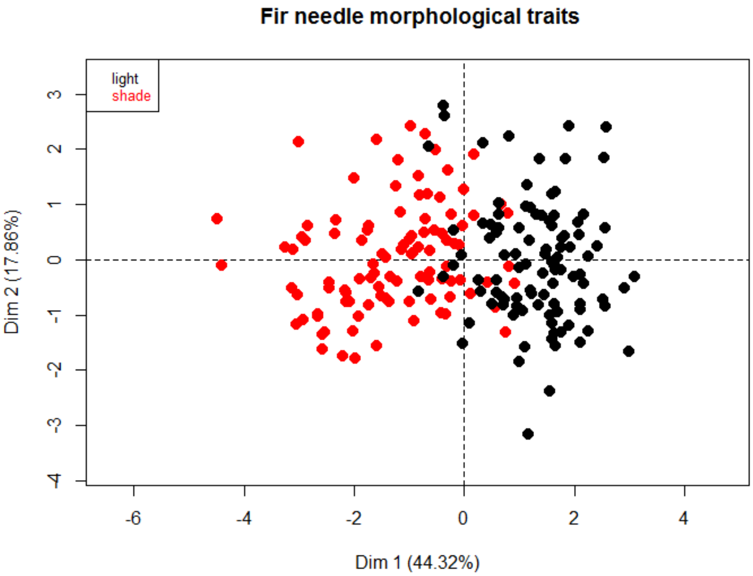

**Figure 2** Plot of individual trees for the first two PCA axes for morphological traits of light and shade needles

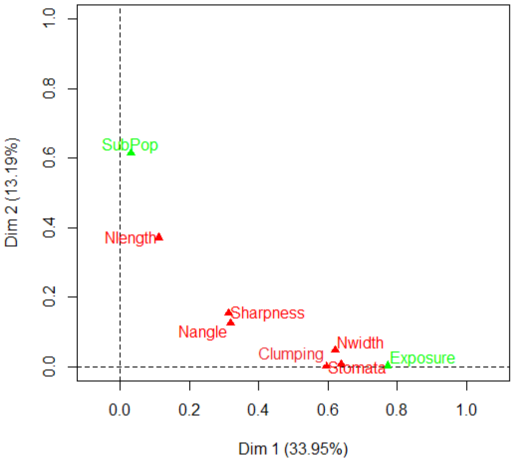

**Figure 3** MFA plot showing the correlation of needle variables, subpopulations and light exposure as explanatory factors with the two first PCA axes

explaining 32.72% of the total diversity.

When shade and light needles were analyzed together, the PCA plot showed a clearly different grouping pattern for light and shade needles (Figure 2). The multiple factor analysis (Figure 3) showed that the first PCA axis expresses the variation influenced by light exposure (33.95%), while the second PCA axis expressed variation caused by the inclusion of the trees in different subpopulations (13.19%). The morphological traits seemed to be most correlated with irradiation, especially needle width and stomatal density. Only needle length approached the second PCA axis and was probably influenced more by the subpopulation than the shading class.

The morphological clusters that were suggested by the MCMC model followed a different pattern for light and shade needles (Figure 4). In light needles, Ag.Petros was mostly differentiated from the other three subpopulations, while in shade needles Ag.Petros showed similarities with Flaburi; Kyra mostly deviated from the other subpopulations.

The analysis of the iSSR and RAPD markers revealed high diversity within the fir subpopulations in Mt. Parnitha, as 62 out of 63 zones scored were polymorphic. High expected heterozygosity values (*He*) were found both in each subpopulation and in the whole set of individuals. The AMOVA showed that diversity was distributed mainly within subpopulations (96%), while differentiation among them was respectively low (4%). All four subpopulations exhibited similar levels of diversity within subpopulations and the average values over loci of allele number (*N*), number of effective alleles (*Ne*) and expected heterozygosity (*He*) were not significantly different (Table 3). Subpopulation Flaburi had a higher allele number, while subpopulations Ag.Triada and Kyra demonstrated higher diversity measures (*Ne* and *He*).

Genetic differentiation among subpopulations was low, as indicated by the AMOVA and the low $G_{st}$ value ($G_{st} = 0.064$). The genetic distances produced a UPGMA tree showing that Ag.Petros and Flaburi were more closely related, while the other subpopulations were more distant (Figure 5). This was observed as well in results of the DAPC. The PCA loadings of the first 35 components, representing more than 90% of the total genetic diversity, were used to cluster the individuals via a discriminant analysis. Two clearly defined clusters were observed. The distribution of these clusters in the subpopulations (Figure 6) showed that Ag.Petros and Flaburi had a similar pattern, while Ag.Triada and especially Kyra were more deviating from the other two subpopulations. When a MCMC clustering approach was used for the genetic data, six genetic clusters were defined, representing six sets of individuals in HW equilibrium. The distribution of these clusters in the subpopulations (Figure 7) showed a similar outcome as the discriminant analysis; Ag.Petros and Flaburi were similar, while Ag.Triada and Kyra were different from the other two subpopulations and from each other.

The genetic differentiation levels between subpopulations showed a specific grouping pattern (Figures 5, 6, 7). Ag.Petros and Flaburi

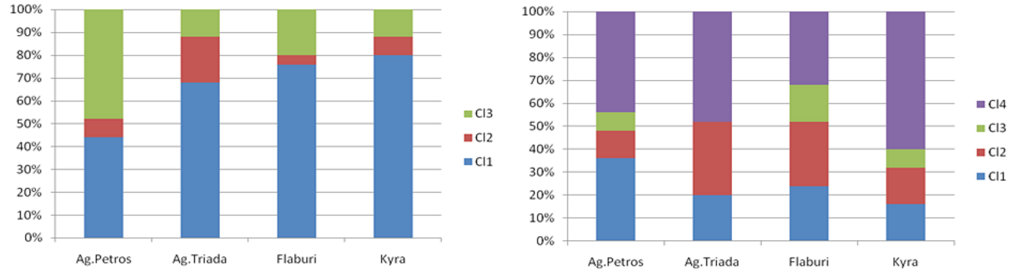

**Figure 4** Distribution of morphological clusters (Cl) in the subpopulations for (left) light and (richt) shade needles

**Table 3** Genetic diversity within fir subpopulations of Mt. Parnes

| Subpopulation | | *N* | *P(%)* | *Na* | *Ne* | *He* |
|---|---|---|---|---|---|---|
| Ag.Petros | Mean<br>(SE) | 24.00 | 87,30 | 1.841<br>(0.056) | 1.542<br>(0.045) | 0.313<br>(0.022) |
| Ag.Triada | Mean<br>(SE) | 23.00 | 87,30 | 1.873<br>(0.042) | 1.560<br>(0.045) | 0.320<br>(0.022) |
| Flaburi | Mean<br>(SE) | 24.00 | 88,89 | 1.889<br>(0.040) | 1.526<br>(0.044) | 0.307<br>(0.022) |
| Kyra | Mean<br>(SE) | 24.00 | 88,89 | 1.873<br>(0.048) | 1.552<br>(0.044) | 0.318<br>(0.022) |
| Total | Mean<br>(SE) | 23.75<br>(0.027) | 88,10<br>(0.46) | 1.869<br>(0.023) | 1.545<br>(0.022) | 0.314<br>(0.011) |

Note. Abbreviations: *N* - number of individuals, *P(%)* - percentage of polymorphic zones, *Na* - mean number of alleles over loci, *Ne* - mean effective number of alleles over loci, *He* - mean expected heterozygosity over loci, SE - standard error.

had a more or less similar structure of genetic diversity, while Ag.Triada and especially Kyra were differentiated. For morphological traits, differences between light and shade needles were observed in terms of differentiation among subpopulations. In shade needles, subpopulations were grouped similarly to the genetic markers (Figure 4); Ag.Petros showed similarities with Flaburi, while Kyra mostly deviated from the other subpopulations. In light needles, differentiation among subpopulations was lower and Ag.Petros was apparently different from the other three subpopulations.

This similarity between genetic markers and morphological traits of shade needles, as far as differentiation at the subpopulation level is concerned, was not observed at the level of individual trees. A Mantel test comparing two dissimilarity tables among individuals, one with genetic distances and one with Euclidian distances for shade needles, showed no correlation ($r$ = -0.062, $p$ = 0.09). The comparison between genetic markers and morphological traits of light needles resulted in even less correlation ($r$ = 0.008, $p$ = 0.424). Only traits of light and shade needles were slightly correlated ($r$ = 0.136, $p$ = 0.006). A MFA using all three data sets (genetic, morphological for light needles and morphological for shade needles) produced a week ordination with the first PCA axis representing only 7.35% of the total variation.

## Discussion

Our results indicate that needle position on

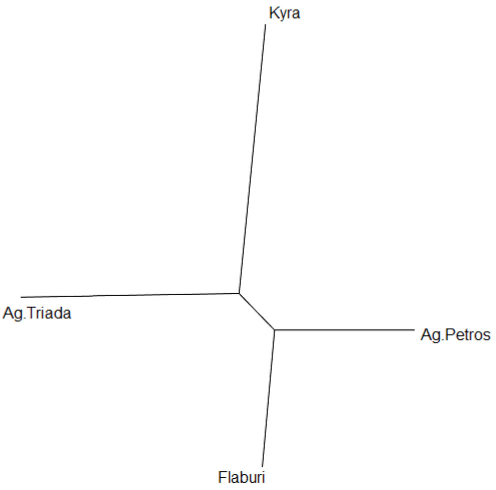

**Figure 5** UPGMA dendrogram based on the Nei unbiased genetic distance among subpopulations (d = 0.01)

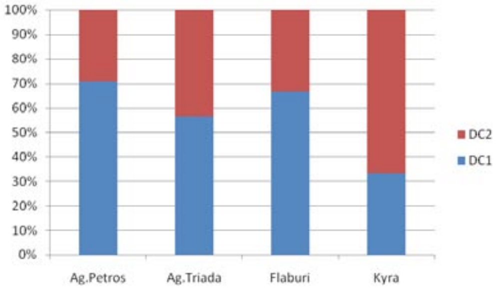

**Figure 6** Distribution of discriminant analysis clusters (DC) in the subpopulations

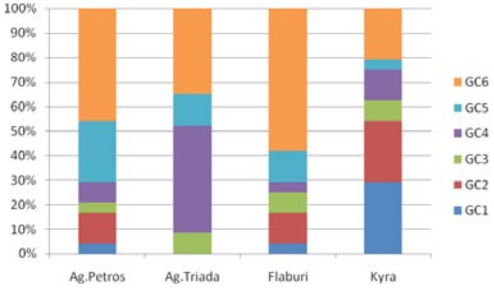

**Figure 7** Distribution of discriminant analysis clusters (DC) in the subpopulations

the tree crown and therefore solar radiation is an important factor influencing needle size, shape and position of needles on the shoots in *A. cephalonica* on Mt. Parnitha. Light needles were significantly different than shade needles. This result was observed in all subpopulations especially for needle width, needle clumping and stomatal density. Needles exposed to direct light were wider, had more stomata on the upper side and were forming shoots with “brush-like” structure. These results reveal that *A. cephalonica* trees apparently follow a different light harvesting strategy, which is well documented for other coniferous species (e.g. Carter & Smith 1985, Oker-Blom et al. 1991, Stenberg et al. 1999, Gebrauer et al. 2011).

The higher width observed under direct light implies an increase in the total projected needle area (e.g. Lewis et al. 1999, Sellin 2001) which in turn deduces into increment of light harvesting efficiency for maximization of photosynthesis. This requires the provisioning of greater amount of $CO_2$ into the palisade parenchyma. The last one comes in accordance with the higher stomata number under light. The increased number of stomata gives the potential for higher $CO_2$ diffusion in the photosynthetic apparatus and can increase the net assimilation rate. Although the rise in needle width with increasing light availability is commonly observed in conifers (Niinemets & Kull 1995, Parker & Mohammed 2000, Niinemets et al. 2001, Palmroth et al. 2002, Wainhouse et al. 1998, Niinemets & Lukjanova 2003, Niinemets et al. 2007), there are also references about the opposite trend (e.g. Duchesneau et al. 2001, Richardson et al. 2001). For example Youngblood & Ferguson (2003) report that the different light environments did not affect needle width or length in *Abies grandis, A. lasiocarpa* and *Picea engelmanii* but affected positively the stomatal density in *A. lasiocarpa* and *P. engelmanii*.

Similar to width, an increase in needle length was also observed, even in a less extent, under direct light conditions within the same tree in our study. Yet, this trend was not reported in studies of other fir species (Morgan et al. 1983) or other conifers (Cregg et al. 1993, Metslaid et al. 2007). Thus, a species specific behavior may exist in both needle width and length variation relative to light availability in *A. cephalonica*, but this needs to be further investigated.

Differences between light and shade needles in needle clumping and needle angle are probably the outcome of the different strategies for light harvesting described in this study. Needles located in shoots on the outer part of the canopy tend to increase the diffusive light to the interior of the canopy while needles in the shaded shoots try to maximize the incoming light interception (Ishii et al. 2012). Needle clumping is an adaptive trait which correlates with increasing light availability (Niinemets et al. 2002). According to Niinemets et al. (2006) needle clumping can be considered as a balance or tradeoff between light harvesting and photosynthetic biomass accumulation. The higher needle clumping observed in our study results to the increase of the diffusive light in the interior of the canopy giving an advantage to the shade needles to make a better exploitation of the available diffusive light and increase carbon gain through more efficient photosynthesis (e.g. Stenberg 1996, Mitchell 1998, Alton et al. 2007).

Under intense irradiation, leaves perpendicular to the sunlight usually are prone to photoinhibition. Plants need to avoid this phenomenon and modify their leaf to shoot angle according to light conditions (Metslaid et al. 2007). The angle between the needle and the axis of the twig becomes smaller with increasing irradiation (e.g. Niinemets et al. 2002). This protects the leaf photosynthetic apparatus and in combination with a higher needle clumping in light shoots, it increases the possibility of a reflected photon from a needle surface to interact again with another needle in the same shoot (see also Smolander & Stenberg 2003). Additionally, the lower needle to axis angle in light shoots protects them from winter embolism phenomena, common in the high altitudes near the tree line (Mayr et al. 2003).

There was variation among subpopulations in needle morphology, where different trends appeared under different irradiation levels. In light needles, differentiation among subpopulations was lower and the subpopulation Ag.Petros, located on the north slopes of the mountain, was different from the other three subpopulations that had a different aspect. Light needles in Ag.Petros were wider and the needle to axis angle was smaller than in the other subpopulations. Variation in needle morphology with geographical aspect has been also documented by Zobel (1973) in intergrading *A. grandis* and *A. concolor* populations. Northern aspects usually receive direct light for shorter time during the day. Trees seem to adapt to the reduced light availability by increasing the size of light needles and changing the needle to axis angle as observed in our study.

Differentiation among subpopulations was higher in shade needles, in traits such as needle width, needle sharpness and stomatal density. Subpopulations Ag.Petros and Flaburi had similar patterns in these traits, while Ag.Triada and especially Kyra were much differentiated. Since there are no studies comparing fir populations and species in morphological traits of shade needles, no conclusion can be drawn connecting these differentiation patterns with specific adaptation strategies. Indeed, the relationships between light and morphological parameters are not always species specific and may vary for a certain species among different environments or be influenced by other environmental factors (Wright et al. 1998)

Our results also indicate that the only trait with a significant interaction between subpopulation and solar radiation was needle width. Indeed, the plasticity of individual trees in this trait varied largely among subpopulations. Differences between light and shade needles were larger in Ag.Petros and Flaburi and smaller in Ag.Triada and Kyra. This trend was also observed in needle sharpness and stomatal density, but was not statistically significant. It seems that the plasticity of firs in needle width, sharpness and stomatal density is larger in areas where radiation is low, due to aspect and topography. Plasticity in needle size and shape is probably related with adaptive strategies of trees that enables them to survive under low irradiation, as reported for species with different tolerance to shade (Grassi & Giannini 2005, Canham 1988, Chen et al. 1996, Claveau et al. 2002, Givnish 1988, Williams et al. 1999).

The results from the analysis of DNA markers reveal that the genetic diversity within the total fir population in Mt. Parnitha and within the four subpopulations was high, in comparison to the average diversity found in populations of outcrossing perennial plant species, using the same type of genetic markers (Nybom 2004, Ai 2014). Despite the high diversity observed in our study, previous studies employing isozyme markers describe the fir population of Mt. Parnitha as one of the less diverse among Greek fir populations (Scaltsoyiannes et al. 1991, Drouzas 2000). However, these studies were based on samples collected from provenance trials. In this study we collected material from the whole distribution of *A. cephalonica* in Mt. Parnitha, while the material used in establishing the provenance trials might have originated from a part of the distribution, a fact that may explain the low diversity reported.

The high levels of diversity indicate that the adult fir trees on Mt. Parnitha were abundant enough to maintain diversity and that gene flow within the broader population and thus among the subpopulations studied was high. This agrees with the low differentiation levels measured among the subpopulations of this study. Despite the adverse environmental conditions, the problems reported in tree health (Heliotis et al. 1988, Tsopelas et al. 2001, Tsopelas et al. 2004) and the lack of regeneration (Apatsidis et al. 2005, Ganatsas et al. 2012), subpopulations Ag.Triada and Kyra, growing on the southern side of the mountain, showed higher diversity levels than the other two subpopulations studied. Unfortunately, these two populations showing higher diversity were burnt completely during the wildfire of 2007. Significant to high levels of genetic diversity have been reported for other mountainous populations of conifers in the Mediterranean, growing under adverse environmental conditions, such as *Cupressus sempervirens* in Crete (Papageorgiou et al. 1994), *Cedrus brevifolia* in Cyprus (Eliades et al. 2011), *Abies nebrodensis* in Sicily (Vicario et al. 1995; Parducci et al. 2001), *Abies cilicica* in Lebanon (Awad et al. 2014), *Abies* species in west Mediterranean (Terrab et al. 2007) and *Picea omorika* in Serbia (Aleksic & Geburek 2014).

While differentiation among subpopulations in our study was found low and the genetic distances among them were low as well, a specific grouping pattern was observed at the subpopulation level. Ag.Petros and Flaburi were genetically similar, while Ag.Triada and Kyra were different from the other two subpopulations and from each other. Possibly, this trend can be explained geographically, since subpopulations Ag.Petros on the north side and Flaburi on the east side of the mountain are closer to each other and seem to be more connected, as they are part of the same dense fir forest. Ag.Triada and Kyra are located on the southern side of the mountain and the forest connecting them with the other subpopulations is sparse. This specific grouping trend is repeated in the results of several analyses in our study, despite the fact that differentiation is generally low.

The results of our study further indicate a similar grouping pattern for genetic markers and morphological traits measured in shade needles. It seems that the size and shape of fir light needles and their position on the shoots on the outer side of the crown follow a specific pattern connected with the ability to avoid impacts of direct intense light and to better utilize the diffusive light in the inner part of the crown. This response of needles exposed to light is apparently universal across several tree species and is connected with the achievement of minimal saturation costs that creates a more or less uniform morphological pattern (e.g. Niinemets & Kull 1995, Niinemets et al. 2006, Ishii et al. 2012). Shade needles are obviously less influenced by radiation and seem to better express inherited differences among subpopulations. If this is the case, morphological diversity among subpopulations in shade needles is possibly influenced by connectivity between forest stands and gene flow, the same factors that seem to influence the neutral genetic markers in this study. To our knowledge, there have not been any studies so far comparing diversity and differentiation patterns between light or shade needle morphological traits and genetic markers in conifer populations. However, irradiation may not be the only environmental factor influencing needle morphology and the differences among subpopulations observed in this study may have more complex adaptive or non adaptive explanations (Wright et al. 1998).

## Conclusions and perspectives for restoration

The wildfire of 2007 has burnt the largest part of the fir forest, including subpopulations Ag.Triada and Kyra that were studied here. They were located on the south slopes of the mountain, growing under adverse environmental conditions and facing health problems and low regeneration (Heliotis et al. 1988, Tsopelas et al. 2004, Apatsidis et al. 2005). Our results show that these subpopulations had the highest genetic diversity and were also most differentiated than the other two that survived (Ag.Petros and Flaburi). Since fir does not regenerate after fire, a large part of the genetic diversity of the fir population on Mt. Parnitha must be considered as lost. According to our results, the subpopulations studied were probably adapted to the diverse environmental conditions of their sites. Thus, the genetic diversity lost was including adaptations to the adverse conditions of the sites where firs were growing, on the south side of Mt. Parnitha.

The currently existing subpopulations Ag.Petros and Flaburi showed high diversity as well, although lower than the one of the burnt subpopulations. Additionally, the levels of gene flow have been probably enough to maintain a low differentiation and a more or less uniform gene pool across the fir forest. This means that a large part of the genetic diversity of the burnt trees still exists in the surviving subpopulations. Since the surviving 1/3 of the original fir forest is large enough and in good condition, it may well provide the necessary reproductive material for restoration of the burnt part. We suggest that seed should be gathered during masting years in a systematic procedure, so that genetic diversity of the existing population will be best represented in the seedlings of the next generation (see also Ganatsas et al. 2012). It would be advisable that seedlings should be grown and screened in a local nursery, so that the trees that will be planted will have increased possibilities to be adapted on the specific non-favorable conditions of the site. Importing seedlings originating from other fir forests in Greece should be avoided, since the fir population of Mt. Parnitha has shown to be discrete compared to all other Greek fir populations. Finally, an ex-situ preservation approach is also essential, in order to maintain a diverse and differentiated gene pool of Greek fir as well as for securing reproductive material to meet future re-establishment needs.

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