

Dering M.<sup>1</sup>, Sękiewicz K.<sup>1</sup>, Boratyńska K.<sup>1</sup>, Litkowiec M.<sup>1</sup>, Iszkuło G.<sup>1, 2</sup>, Romo A.<sup>3</sup>,  
Boratyński A.<sup>1</sup>

**Genetic diversity and inter-specific relations of western Mediterranean relic *Abies* taxa  
as compared to the Iberian *A. alba***

<sup>1</sup>Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland,

<sup>2</sup>University of Zielona Góra, Faculty of Biological Sciences, Prof. Z. Szafrana 1, 65-516,  
Zielona Góra, Poland

<sup>3</sup>Consejo Superior de Investigaciones Científicas, Institute of Botany, Barcelona, Spain

Corresponding author: [mdering@man.poznan.pl](mailto:mdering@man.poznan.pl), phone: + 48618170033, fax: +48618170166

**Abstract**

Several *Abies* species are currently present in the Mediterranean Region and most of them are endemits and Tertiary relicts. Using six nuclear microsatellites, we studied the genetic structure and inter-specific relationships among West Mediterranean firs, *A. pinsapo* (Spain), *A. maroccana* and *A. tazaotana* (Morocco). Based on the hypothesis that *A. pinsapo* could historically exchange genes with *A. alba* growing in the Pyrenees *via* secondary contact, we investigated the level of genetic admixture between these species using a Bayesian approach. The studied populations showed moderate genetic diversity (mean  $H_E = 0.598$ ) and a high level of genetic differentiation ( $F_{ST} = 0.225$ ) that was especially pronounced between *A. alba* and African firs. All populations experienced a strong bottleneck effect that was likely induced by climatic changes occurring in the west Mediterranean during the last glacial cycle and the Holocene. According to Bayesian clustering, both African taxa grouped together in a single cluster, the two *A. pinsapo* populations formed a second cluster, and two additional

clusters were detected within *A. alba*. Our results indicate that *A. tazaotana* is genetically very close to *A. maroccana*, and hence these two taxa should probably not be considered as separate species. We found no genetic admixture between *A. pinsapo* and *A. alba* and only minor between *A. pinsapo* and African fir populations suggesting an isolation effect of the Gibraltar Strait. Current limited distributions of firs in the Mediterranean Region together with changing climate may lead to further deterioration of the genetic diversity levels. Hence, future efforts should focus on monitoring the demography and genetic threats to existing populations.

**Keywords:** *A. pinsapo*, *A. alba*, genetic diversity, phylogeography, Gibraltar Strait, West Mediterranean firs

## ***Introduction***

In Europe, the genus *Abies* Mill. has a circum-Mediterranean distribution. *Abies alba* Mill. is the only species with a widespread European range, while the remaining taxa are mostly allopatric, with the distributions often confined to very limited areas (Shütt, 1991). The striking feature of the firs is their high level of endemism, being the evolutionary legacy of the complex climatic and geological history of the Mediterranean Region (Thompson, 2004; Linares, 2011). Additionally, large morphological variability and perplexing phylogenetics due to inter-specific hybridization make the systematic classification of the Mediterranean firs still disputable (Parducci and Szmidt, 1999; Scaltsoyiannes et al., 1999; Parducci et al., 2001). Based on the bracts of the cones, firs are classically divided into two main groups: the archaic firs and the modern firs (Liu, 1971). Species from the first group are morphologically characterized by enclosed bracts while species from the second group have protruding bracts.

In the Western Mediterranean, a few fir taxa are present. On the Iberian Peninsula, *A. alba* being the representative of modern firs (section *Abies*) occurs in the Pyrenees, practically out of the Mediterranean Region (Bolòs and Vigo, 1984; Villar et al., 1997). The archaic firs (section *Piceaster* Spach) are represented by *A. numidica* Carr growing in northern Africa (Algeria) and *A. pinsapo* Boiss. agg. occurring on both the Iberian Peninsula and in northwestern Africa (Morocco) (Farjon, 2010). The phylogenetic relationships among the populations of *A. pinsapo* agg. are complex and, hence, their taxonomical status is still unresolved. Liu (1971) and Farjon (2010) recognized within *A. pinsapo* agg. two varieties – *A. pinsapo* var. *pinsapo* noted in southern Spain and *A. pinsapo* var. *maroccana* (Trab.) Ceballos et Bolaño growing in northern Morocco. The latter taxon, however, is not homogenous and a third variety – *A. pinsapo* var. *tazaotana* (S. Còzar ex Villar) Pourtet is sometimes distinguished. Arista and Talavera (1994), based on morphological analysis, treats each of the three varieties as a separate species, *A. pinsapo*, *A. tazaotana* Villar and *A.*

*maroccana* Trab. Due to lack of taxonomic consensus, we adopted the classification by Arista and Talavera (1994) in this work.

The modern geographic ranges of these Western-Mediterranean archaic firs have a relict character and are very confined, which may be critical for their future persistence (Arista et al., 1997). In general, population decline is observed due to fires, overgrazing, pests and timber harvesting (Esteban et al., 2010). The climatic trend in this region, manifested by rising temperatures and decreasing precipitation, threaten the future existence of fir forests, especially those in Spain (Esteban et al., 2010). Currently, *A. pinsapo* is present as three populations in the Sub-Betic Mountains in southern Spain (e.g., Esteban et al., 2010), *A. maroccana* is noted from a few stands in the Rif Mts. (Fig. 1; Achhal, 2002), while *A. tazaotana* has been reported only as a single stand in the Tazaot Mt., also in the Rif Mts. (Fig. 2). According to data from 2008, the *A. tazaotana* population was covering a 493-ha area spanning from 1,400–1,800 m of Jbel Tazaote (Esteban et al., 2010). All these taxa are recognised as endangered in the IUCN Red List of Threatened Species (<http://www.iucnredlist.org>).

The occurrence of the silver fir in the Pyrenees is historically related to the Pleistocene refugium that existed there during the last cold stage (Liepelt et al., 2002; Terhüne-Berson et al., 2004; Médail and Diadema, 2009). However, these populations were unable to expand after glacial retreat and, thus, left no legacy in the present gene pool of the species in the wide-European range, similar to Scot pines that have persisted in that region (Terhüne-Berson et al., 2004; Pyhäjärvi et al., 2008; Liepelt et al., 2009). The distinction between the Iberian silver fir populations of the remaining European stands were noted with allozyme markers by Konnert and Bergman (1995). Additional evidence came from Vendramin et al., (1999) who, using paternally inherited cpSSR markers, not only confirmed the distinct character of the Pyrenees population, but also revealed its very low genetic diversity suggesting severe

bottleneck.

Some studies have revealed unexpectedly closer inter-specific relationships between archaic firs and silver firs representing the modern firs, than among the species from the archaic fir group itself (Scaltsoyiannes et al., 1999; Liepelt et al., 2010; Aguirre-Planter et al. 2012). The explanations assume, (1) historical gene flow, perhaps a result of secondary contact at the end of the Pliocene and early Pleistocene (Alba-Sánchez et al. 2010) or (2) a common ancestry. The latter hypothesis is very likely if we consider a monophyletic origin of the genus *Abies* (Scaltsoyiannes et al., 1999; Linares 2011). Liepelt et al. (2010) have revealed genetic affinities between Spanish *A. alba* and two African species, namely *A. numidica* and one of the two Moroccan firs referred by the authors as Moroccan *A. pinsapo* (unfortunately we cannot resolve whether it refers to *A. tazaotana* or *A. maroccana*). Scaltsoyiannes et al. (1999) and Aguirre-Planter et al. (2012: Fig. 2) also suggested close relationships between Spanish *A. pinsapo* and *A. alba* being morphologically and taxonomically distinct (Liu 1971; Farjon 2010). Furthermore, Jaramillo-Correa et al. (2010) reported that *A. maroccana* and *A. numidica* share the mtDNA haplotype that was genetically related to mtDNA haplotype present in *A. alba*.

Here, we used nuclear microsatellites (nSSR) in order to describe the distribution of genetic diversity in Western-Mediterranean archaic firs and *A. alba*. Specifically, our aim was to investigate the genetic relations between Pyrenean silver fir and the west-Mediterranean *A. pinsapo* agg.. A biparental mode of inheritance and high polymorphism of nSSR markers make them a suitable tool in studies of genetic admixtures. Also, using Bayesian methods, we wanted to identify clusters of individuals belonging to different gene pools that may have taxonomical implications and would help to clarify the genetic relationships among the archaic firs.

## Material and methods

### *Study sites and genetic analysis*

Material was sampled from 30 randomly distributed individuals per taxon (separated > 50 m to avoid sampling relatives) from two Spanish populations of *A. pinsapo*, three *A. alba* and two Moroccan endemics, *i.e.* *A. tazaotana* and *A. maroccana* (Table 1, Fig. 3), from 207 individuals in total. Vouchers of individuals from each population were deposited in herbaria of the Institute of Dendrology (KOR) or the Institute of Botany in Barcelona (BC). Total genomic DNA was extracted from needles according to Dumolin et al. (1995).

Nuclear genetic diversity was assessed using six nuclear microsatellite markers (nSSR): SFb5, SF333, SF1, SF239, SF78 and SFb4 (Cremer et al., 2006). The PCR was run in single multiplex reactions using the Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with ca. 30 ng of template DNA. The PCR thermal profile started with a denaturation step at 95 °C for 15 min., followed by 10 touchdown cycles at 94 °C for 30 s, 60 °C for 30 s (-1 °C/ cycle), 72 °C for 40 s, 30 cycles at 94 °C for 30 s, 50 °C for 50 s, 72 °C for 40 s, and a final extension at 72°C for 7 min. Analysis of fluorescently labelled PCR products was performed on an ABI PRISM 3130 genetic analyzer with the GeneScan 500-LIZ size standard (Applied Biosystems). Genotypes were scored using the GeneMapper vs. 4.0. software package (Applied Biosystems).

### *Data analysis*

#### *Genetic diversity and differentiation*

Linkage disequilibrium for all microsatellite loci pairs in each population and standard multilocus parameters of genetic diversity, including average number of alleles per locus ( $A$ ), observed heterozygosity ( $H_O$ ), and unbiased expected heterozygosity ( $H_E$ ) were calculated using the Fstat v. 2.9.3 (Goudet, 2001). Both null alleles and inbreeding lead to excess homozygosity in a population and if either of them is not accounted for during estimation,

there is an overestimation of the other. Using INEst 1.0 (Chybicki and Burczyk, 2009), we calculated corrected values of  $F_{IS}$  along with the frequency of the null alleles, which gives a more reliable overview of the factors affecting the homozygosity level in populations. Deviation of genotypic frequencies from Hardy-Weinberg proportions was tested using the exact test based on the Markov Chain Monte Carlo (MCMC) algorithm using GenePop v. 4.0 (Raymond and Rousset, 1995; Rousset, 2008).

Global genetic differentiation among populations was assessed by computing the  $F_{ST}$  of Weir and Cockerham (1984) with the program Fstat (Goudet, 2001). Pairwise  $F_{ST}$  was calculated in Arlequin 3.11 for all pairs of populations and tested with 3,000 random permutations (Excoffier et al., 2005). Null alleles may lead to an overestimation of  $F_{ST}$ , thus, we used FreeNA with excluding null allele correction (ENA) to estimate the global and pairwise  $F_{ST}$  and tested for significance with 1,000 random permutations (Chapuis and Estoup, 2007).

In searching the geographic patterns, we used a Bayesian approach as implemented in Geneland 3.2.4, which reveals the population genetic structure by placing sampled individuals in  $K$  numbers of clusters with optimal Hardy-Weinberg equilibrium and linkage equilibrium (Guillot et al., 2008). In Geneland we used *Spatial* and *Allele correlated* options with the *Null Allele* model. The real  $K$ -value in Geneland is given as the modal value of posterior distribution of number of populations along the chain that is plotted on the histogram. Estimates for georeferenced data were obtained after 200,000 iterations (saving every 50<sup>th</sup>) in 100 independent runs. The hierarchical distribution of genetic variation among groups defined by Geneland was further investigated using analysis of molecular variance (AMOVA) conducted in Arlequin tested with 1,000 random permutations (Excoffier et al., 2005). In order to infer the range of genetic admixture, we ran Geneland analysis with a fixed number of clusters inferred in the first analysis according to settings described above. The genetic

relationships among populations were further analysed by principle coordinate analysis (PCA) using GenAlEx v. 6 (Peakall and Smouse, 2006). A Mantel matrix correlation (1967) was calculated to test for isolation by distance (IBD). We used Slatkin's (1995) linearized  $F_{ST}$  ( $F_{ST}/(1-F_{ST})$ ) with ENA correction as a measure of genetic distance between pairs of populations and pairwise geographic distance [ $\ln(\text{geographic distance in km})$ ]. The significance of correlation was tested with 1,000 random permutations in PopTools v. 3.2.3. (Hood, 2010). Finally, we compared  $F_{ST}$  and  $R_{ST}$  in order to infer the phylogeographic structure (Hardy et al., 2003).  $R$ -statistics are the analogue to  $F$ -statistics but are based on allele sizes instead of allele identity.  $R_{ST}$  assumes diversity resulting mainly from the mutation process according to the Stepwise Mutation Model (SMM). If  $R_{ST} > pR_{ST}$  is statistically significant, then the stepwise-like mutation at microsatellite loci contribute significantly to population differentiation. The significance of the SMM in shaping the genetic diversity was verified with a permutation test (10,000 permutations) in SpaGeDi 1.2 (Hardy and Vekemans, 2002).

In order to infer the possible "bottleneck" effect in demographic history of the studied populations, the MSVAR v.0.4.2 was used (Beaumont, 1999). The model assumes that the demographic history of a single population of size  $N1$  consists of coalescence and mutation events going back, from time  $Ta$  (in generations) to the current time, until the most recent common ancestor,  $N0$ . The methods are based on the set of demographic parameters,  $r = N0/N1$ ,  $tf = a/N0$  and  $\theta = 2N0\mu$  ( $N0$  – current effective population size,  $N1$  – the ancestral effective population size preceding the demographic change,  $\mu$  – mutation rate). The advantage of this method is that required priors on demographic parameters are scaled ( $r$  and  $tf$ ), which can be proposed more easily than priors for the natural demographic parameters (i.e.,  $N0$ ,  $N1$ ) (Girod et al., 2011). The analysis was conducted assuming the exponential growth model, with rectangular priors for  $\log(r)$ ,  $\log(tf)$  and  $\log(\theta)$  bounded on -5 and 5. The MCMC was run with the  $10^9$  steps at thinning at  $3 \times 10^4$  and excluding the first 10% of steps

to avoid the dependence of final results on starting settings. Additionally, the effective population size,  $N_{e(LD)}$  was estimated based on the linkage disequilibrium method using LDNe software (Waples and Do, 2008). Coalescent time was inferred from the MSVAR  $tf$  ratio (generation time) since the occurrence of the “bottleneck” in particular populations was converted to absolute time (years) according to a formula of Lande et al. (2003),  $T = \alpha + [s/(1 - s)]$ , where  $T$  denotes generation time, while  $\alpha$  and  $s$  are the age in maturity (generative reproduction ability) and the annual survival rate, respectively.

## Results

### *Genetic diversity and differentiation*

No evidence for linkage disequilibrium was detected and all SSR loci were polymorphic. Gene diversity ( $H_E$ ) ranged from 0.478 (ES\_3, *A. alba*) to 0.670 (ES\_4, *A. pinsapo*), and the average number of alleles per locus ( $A$ ) ranged from 3.8 (ES\_2, *A. alba*) to 7.5 (ES\_4 and ES\_5, *A. pinsapo*) (Table 2). Private alleles were detected in all populations. Their frequency ranged from 1.8% (ES\_4) up to 40.4% (MO\_1). A substantial frequency of null alleles was found in all studied populations, and it ranged from 5.1% to nearly 13%, with an average of ca. 10% (Table 2). Because null alleles are responsible for increasing the level of homozygosity, genotypic proportions deviated significantly ( $p < 0.001$ ) from Hardy-Weinberg expectations, and high values of inbreeding coefficient ( $F_{IS}$ ) were noted (Table 2). However, estimation of  $F_{IS}$  with ENA correction resulted in a substantial decrease of the  $F_{IS}$  value in most of the stands (Table 2).

A considerable level of statistically significant differentiation was found among studied populations as global  $F_{ST} = 0.225$  ( $p < 0.01$ ) and *A. pinsapo* agg. populations were more diverged ( $F_{ST} = 0.096$ ,  $p < 0.01$ ) in comparison to *A. alba* populations ( $F_{ST} = 0.029$ ,  $p < 0.05$ ). Accordingly, pairwise  $F_{ST}$  values (Table 3) were significant ( $p < 0.05$ ), except for two

Spanish populations of *A. pinsapo*, ES\_4 and ES\_5. The highest level of pairwise differentiation ( $F_{ST} = 0.387$ ) was observed between *A. maroccana* and population ES\_3 of *A. alba*. After ENA correction, global and pairwise  $F_{ST}$  decreased but not significantly (global  $F_{ST}^{ENA} = 0.214$ ,  $p < 0.05$ ); this indicates a marginal effect of null alleles on estimations of the genetic differentiation.

MSVAR indicated a historical decline in all studied populations (Table 4). According to calculations, the ancestral effective population sizes ( $N_I$ ) were larger than the values of present effective population sizes suggesting wider distribution in the past. The generation time estimated for firs was ca. 207 years based on the assumed annual survival rate of 0.993 and maturity time of ca. 65 years. This allowed for an estimation of a very approximate absolute time of when the decline of populations has started (Table 4). The inferred times of population decline covers the period starting ca. 56,500 years ago until ca. 3,700 years ago, which is the second half of the upper Pleistocene (0.126–0.0117 Ma).

#### *Geographic population structure*

Using Bayesian clustering conducted with Geneland with georeferenced data, we found that seven populations formed optimal  $K = 4$  distinct genetic groups (Fig. 3). Populations of *A. tazaotana* (MO\_1) and *A. maroccana* (MO\_2) were in Cluster I, populations of *A. pinsapo* (ES\_4, ES\_5) were in Cluster II, while three silver fir populations were split into two further clusters: Cluster III - ES\_1 and populations ES\_2 and ES\_3 in Cluster IV. Accordingly, AMOVA revealed that a statistically significant proportion of genetic variation (22.4%,  $p = 0.02$ ) was among these three groups. The admixture analysis revealed extensive intermingling among *A. alba* populations, minor among *A. pinsapo* agg. and no signs of gene exchange between silver fir and any of the archaic fir species (Fig. 3).

The ordination of all *Abies* populations according to PCA largely supported the results

of cluster analysis (Fig. 4). The first principle coordinate (71.6% of the total variation) split the populations into two groups. In the first one, all populations of silver fir were grouped, while the in the second one populations of *A. pinsapo sensu stricto* were clustered. The second coordinate (13.3% of the total variation) revealed further separation pointing mainly to a distinct character of both African taxa (*A. maroccana* and *A. tazaotana*) and very close relationships among *A. pinsapo* populations.

The correlation between nuclear genetic differentiation and geographic distance showed that IBD pattern among the studied populations was very strong ( $R^2 = 0.869$ ,  $p = 0.005$ ). Global genetic differentiation taking into account the allele sizes,  $R_{ST} = 0.46$  was significantly higher than  $pR_{ST} = 0.14$  based on allele identity ( $p = 0.001$ ) suggesting a significant influence of the stepwise-like mutation process on the pattern of genetic differentiation.

## ***Discussion***

### *Genetic diversity and differentiation*

Almost all of the studied populations showed a high level of genetic diversity. The exception was population ES\_3 of *A. alba*, showing the relatively lowest variability. It is perhaps related to the relic and isolated distribution of this species on the Iberian Peninsula and to the bottleneck effects detected. In general, in the Mediterranean Region woody species show a higher level of differentiation in comparison to the trees in the temperate zone (Fady-Welterlen, 2005; Hampe and Petit, 2005; Douaihy et al., 2011; Juan et al., 2012). It is mostly explained by their particular distributional patterns, *i.e.* persistence in the same regions during the Pleistocene glacial periods in fragmented and/or limited ranges. The level of overall genetic differentiation reported for the studied firs was very high ( $F_{ST} = 0.225$ ), but this mostly

reflects a substantial genetic divergence between *A. alba* and the archaic firs due to extended microevolution processes leading to final diversification within *Abies* (Linares, 2011).

All the populations studied went through a strong bottleneck, and – according to calculations – the ancestral population sizes were much greater (Table 4). Other evidence also confirms that the modern distribution of *A. pinsapo*, *A. maroccana* and *A. tazaotana* is far less than that in the past: palaeobotanical and palaeoclimatic data, together with distributional modeling, suggest that the forest ecosystem was more widespread during the Pliocene in the Mediterranean Region (van Andel and Tzedakis, 1996; Alba-Sánchez et al., 2010).

The estimated time of population decline episodes (starting ca. 56,500 years ago until ca. 3,700 year ago) might be linked to various considerable palaeoclimate changes in the studied region and cover: 1) the second half of the Late Pleistocene for the African populations, 2) the Last Glacial Maximum (LGM) and the transition between the last glacial period and Holocene for the Pyrenees population of *A. alba*, and 3) the mid- to late-Holocene for the population of *A. pinsapo*. The palynological studies of the Alborán Sea (western part of the Mediterranean Sea) sediments showed the large sensitivity of Mediterranean vegetation to the environmental conditions of the glacial period and the millennial oscillation of the Holocene climate (Sánchez Goñi et al., 2002; Fletcher and Zielhofer, 2013). The reduction of forest ecosystems and expansion of semi-desert vegetation was associated with abrupt changes in the Mediterranean climate during the last glacial period associated with Heinrich Events (HEs; van Andel and Tzedakis, 1996; Sánchez Goñi et al., 2002; Roucoux et al., 2005; Fletcher and Sánchez Goñi, 2008; Schmidt et al., 2012). HEs, being the fresh water fluxes into the North Atlantic, were causing a collapse in the thermohaline circulation. The result was a substantial decrease of sea surface temperature leading to Mediterranean palaeoclimate rapid cooling and aridification (Cacho et al., 1999; Sánchez Goñi et al., 2002; Martrat et al., 2004; Fletcher and Sánchez Goñi, 2008). Hence, the demographic collapse noted for silver fir

ES\_1 populations in the Pyrenees might reflect the severe environmental conditions during HE1 (Fletcher and Sánchez Goñi, 2008). Two remaining dates of bottleneck events inferred for ES\_2 and ES\_3 may be linked to the Younger Dryas (Pons and Reille, 1988; Fletcher and Sánchez Goñi, 2008). Additionally, data from the Alborán Sea sediments indicate several incidents of forest community declines in the early Holocene and mid- to late-Holocene in the Iberian Peninsula (Fletcher and Zielhofer, 2013). The late-Holocene decline of *A. pinsapo* populations was probably related to a dry episode occurring ca. 5.4–4.5 cal. ka BP (Fletcher and Zielhofer, 2013).

With regard to the palaeoclimate of the North African region, studies indicate that since the onset of the Pleistocene, ca. 115 ka, the North-African climate was progressively entering a period of hyper-arid conditions (Carto et al., 2009) that have posed a real challenge for the North-African environment and almost certainly pushed out the early humans from Africa (Carto et al., 2009). The aridity of the North African climate fully established around 75 ka (Blome et al., 2012) and narrowed the woody vegetation into mountain regions that could supply the required humidity, whereas the lowlands desert and semi-desert environments prevailed due to Saharan expansion (van Andel and Tzedakis, 1996; Linares, 2011; Blome et al., 2012). One of the dates estimated for the African population decline, 41.2 ka (MO\_2) can be linked to the HE4 as indicated with pollen taxa and marine proxy data retrieved from Alborán Sea marine core MD95-2043 (Roucoux et al., 2005).

#### *Genetic relationships and geographic patterns*

Bayesian clustering and PCA have revealed a high genetic differentiation among taxa of *A. pinsapo* agg., evidenced also with a rather high pairwise  $F_{ST}$  ranging from 0.10 to 0.15. Similarly, Scaltsoyiannes et al. (1999) found considerable divergence among fir species from the section *Piceaster*, *A. cilicica*, *A. numidica* and *A. pinsapo* using isoenzyme markers. The

large genetic distance noted in our investigation between archaic firs and *A. alba* signifies the spatially and temporally complex evolutionary history of species from the genus *Abies* (Linares, 2011). In light of the obtained results, previously reported findings on shared cytoplasmic variability between silver firs and the archaic firs definitely need to be regarded as the legacy of the common ancestry. There are some suggestions of possible overlap between *A. alba* and *A. pinsapo* ranges during the Pliocene (Palamarev, 1989; van Andel and Tzedakis, 1996; Alba-Sánchez et al., 2010). However, the admixture analysis conducted revealed no genetic admixture between both species suggesting that there were no signs detectable at neutral nuclear markers after this hypothetical secondary contact questioning it at all.

Terrab et al. (2007) concluded very low divergence between *A. maroccana* and *A. tazaotana* at cpSSR and has attributed it to intensive pollen mediated gene flow. Morphologically and anatomically, *A. tazaotana* and *A. maroccana* are also very close (Sękiewicz et al., 2013). Our studies based on nuclear microsatellites strongly confirm these results and, consequently, do not support *A. tazaotana* at the species rank. However, the level of genetic differentiation noted among both populations separated by only ca. 10 km is striking and implies a reduced gene flow and/or drift-derived pattern of differentiation. In reference to reduced gene flow, certainly some biological constraints are involved as the geographic isolation can be excluded. The limitations in gene exchange might stem from low pollen production and/or its poor dispersal resulting from the pollen relatively large size (Dybowska-Jachowicz and Sadowska, 2003). Arista and Talevera (1994) reported restricted pollen dispersal capacity in *A. pinsapo*, which can also be true for vicariant Moroccan species. Nevertheless, neither drift nor the limited gene flow favor long-term persistence of the North African fir populations in refer to current climatic trends.

Our investigation revealed minor admixture between Spanish *A. pinsapo* and African firs (Fig. 3). It had an asymmetrical character as only on average 4.8% of *A. pinsapo* genes were shared with African firs, that in turn shared on average 12.7% genes with *A. pinsapo* (Fig. 3). This admixture between species currently occurring on European and African continents might be ascribed to a common ancestry and/or gene flow. Either of these hypotheses is possible, but none can be fully verified with the current data. High genetic divergence between *A. pinsapo* and Moroccan firs at cpSSR markers (Terrab et al., 2007), allowing in conifers for tracing the pollen-mediated gene flow, preclude recurrent airborne gene flow or define it as negligible. The low admixture we have detected at nuclear SSRs suggests exactly the same and supports the hypothesis of a common ancestry. Significant genetic discontinuities across the Strait of Gibraltar noted between the same or closely related taxa are generally explained as resulting from the continents' isolation due to the strait opening at 5.33 Ma (Krijgsman et al., 1999). Prior to this, a Messinian salinity crisis led to almost a complete drying of the Mediterranean Sea following the closure of marine Mediterranean-Atlantic connection induced by tectonic uplift in the area of the Gibraltar arc (Krijgsman et al., 1999). As a result, the inter-continental land bridges appeared and could potentially act as the corridors for genetic exchange. The closure of the Gibraltar Strait certainly has triggered the mammal migration between Africa and Europe (Agustí et al., 2006), though it is skeptical in the case of plant migrations (Thompson, 2004). However, much later, during the last glaciation when sea levels dropped for ca. 120/130 m, emerging islets could facilitate gene exchanges between biota from both sides of the Strait (Otriz et al., 2007). Thus far, phylogeographic patterns induced in the Strait of Gibraltar were described for angiosperm *Quercus ilex* L. (Lumaret et al., 2002), *Pistacia lentiscus* L. (Werner et al., 2002), *Eryngium maritimum* (Kadereit et al., 2005) or *Carex helodes* L. (Escudero et al., 2008) and for conifer *P. pinaster* Aiton (Burban and Petit, 2003), *Juniperus phoenicea* L. (Dzialuk et al.,

2011), *P. nigra* J.F.Arnold, *A. pinsapo*, and *P. pinaster* (Jaromilo-Correa et al., 2010). There are, however, studies contradicting the isolating effect of the Gibraltar Strait and reporting genetic ties between African and European populations e.g., in *J. oxycedrus* L. (Boratyński et al. in print), *Q. suber* L. (Lumaret et al., 2005), *Taxus baccata* and *Pinus halepensis* Mill. (Jaromilo-Correa et al., 2010).

The fir species investigated in this study exemplify the Tertiary heritage as one of the fundamental elements of Mediterranean Region biodiversity. The archaic firs, *A. pinsapo* and *A. maroccana* are the best examples of paleoendemics noted in the Mediterranean making its flora so unique (Thompson, 2004; Linares, 2011). However, their current limited distributions may lead to further deterioration of the genetic diversity due to inbreeding and drift making them more prone to genetic stochasticity processes. Other hazards, resulting mainly from rapid climate changes, including catastrophic fires and pests appearing due to drought stress, may threaten their long-lasting persistence. Hence, monitoring for the demographic and genetic threats should be conducted, and it seems to be especially relevant and urgent for African fir populations. Pyrenees populations of *A. alba*, the species that is widespread across Europe, also require the conservation attention. These populations were shown to have experienced a considerable reduction in historical times resulting in low effective population sizes and low genetic variability.

### **Acknowledgments**

We thank M. Ratajczak, M. Łuczak and A.K. Jasińska for technical support. This research was financed by Ministry of Science and Higher Education in Poland (grant NN303412136) and Institute of Dendrology, Polish Academy of Sciences.

### **Data Archiving Statement**

Data on SSRs genotypes are included as supplemental materials

## References

- Achhal, A. 2002. Pinaceae. In: Valdés, B., Rejdali, M., Achhal el Kadmiri, A., Jury, J.L., Montserrat, J.M. (Eds.), Checklist of vascular plants of N Morocco with identification keys, 1. Universidad de Sevilla, Institut Agronomique et Vétérinaire Hassan II, Rabat, University of Reading, Unstitut Botànic de Barcelona (CSIC-Ajuntament de Barcelona).
- Aguirre-Planter, É., Jaramillo-Correa, J.P., Gómez-Acevedo, S., Khasa, D.P., Bousquet, J., Eguiarte, L.E. 2012. Phylogeny, diversification rates and species boundaries of Mesoamerican firs (*Abies*, Pinaceae) in a genus-wide context. *Mol. Phyl. Sys.* 62, 263–274.
- Agustí, J., Garcés, M., Krijgsman, W. 2006. Evidence for African–Iberian exchanges during the Messinian in the Spanish mammalian record. *Palaeogeog. Palaeoclima. Palaeoeco.* 238, 5–14.
- Alba-Sánchez, F., López-Sáez J.A., Pando, P.B., Linares, J.C., Nieto-Lugilde, D., López-Merino, L. 2010. Past and present potential distribution of the Iberian *Abies* species: a phytogeographic approach using fossil pollen data and species distribution models. *Diver. Distrib.* 16, 214–228.
- Arista, M., Talavera, S. 1994. Phenology and anatomy of the reproductive phase of *Abies pinsapo* Boiss. (Pinaceae). *Bot. J. Linn. Soc.* 116, 223-234.
- Arista, M., Herrera, J., Talavera, S. 1997. *Abies pinsapo* Boiss.: a protected species in a protected area. *Bocconeia* 7, 427–436.
- Beaumont, M.A. 1999. Detecting population expansion and decline using microsatellites. *Genetics* 153, 2013–2029.

- Blome, M.W., Cohen, A.S., Tryon, Ch.A., Brooks, A.S., Russell, J. 2012. The environmental context for the origins of modern human diversity: A synthesis of regional variability in African climate 150,000-30,000 years ago, *J. H. Evol.* doi:10.1016/j.jhevol.2012.01.011.
- Bolòs, O., Vigo, J. 1984. *Flora dels països Catalans*, 1. Editorial Barcino, Balcelona, p. 736.
- Burban, C., Petit, R.J. 2003. Phylogeography of maritime pine inferred with organelle markers having contrasted inheritance. *Mol. Ecol.* 12, 1487–1495.
- Cacho, I., Grimallt, J.O., Pelejerlo, C., Canals, M., Sierro, F.J., Flores, J.A., Shackleton, N. 1999. Dansgaard-Oeschger and Heinrich event imprints in Alboran Sea paleotemperatures. *Paleoceanography* 14:698–705
- Carto, S.L., Weaver, A.J., Hetherington, R., Lam, Y., Wiebe, E.C. 2009. Out of Africa and into an ice age: on the role of global climate change in the late Pleistocene migration of early modern humans out of Africa. *J. H. Evol.* 56, 139–151.
- Chapuis, M-P., Estoup, A. 2007. Microsatellite null alleles and estimation of population differentiation. *Mol. Biol. Evol.* 24, 621–631.
- Chybicki, I., Burczyk, J. 2009. Simultaneous estimation of null alleles and inbreeding coefficients. *J. Hered.* 100, 106–113.
- Cremer, E., Liepelt, S., Sebastiani, F., Buonamici, A., Michalczyk, IM., Ziegenhagen, B., Vendramin, G.G. 2006. Identification and characterization of nuclear microsatellite loci in *Abies alba* Mill. *Mol. Ecol. Notes* 6, 374–376.
- Douaihy, B., Vendramin, G.G., Boratyński, A., Machon, N., Dagher-Kharrat, M.B. 2011. High genetic diversity with moderate differentiation in *Juniperus excelsa* from Lebanon and the eastern Mediterranean region. *AoB Plants* plr003.

- Dumolin, S., Demesure, B., Petit, R.J. 1995. Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theor. Appl. Genet.* 42, 351–359.
- Dzialuk, A., Mazur, M., Boratyńska, K., Montserrat, J.M., Romo, A., Boratyński, A. 2011. Population genetic structure of *Juniperus phoenicea* (Cupressaceae) in the western Mediterranean Basin: gradient of diversity on a broad geographical scale. *Ann. For. Sci.* 68, 1341–1350.
- Dybowska-Jachowicz, S., Sadowska, A. 2003. *Palynology*. W. Szafer Institute of Botany, PAN, Kraków, 411p. [in Polish]
- Escudero, M., Vargas, P., Valcárcel, V., Luceño, M. 2008. Strait of Gibraltar: an effective gene-flow barrier for wind-pollinated *Carex helodes* (Cyperaceae) as revealed by DNA sequences, AFLP, and cytogenetic variation. *Am. J. Bot.* 95, 745–755.
- Esteban, L.G., de Palacios, P., Rodríguez-Losada, Aguado L. 2010. *Abies pinsapo* forests in Spain and Morocco: threats and conservation. *Oryx* 44, 276–284.
- Excoffier, R., Laval, L.G., Schneider, S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinforma.* 1, 47–50.
- Fady-Welterlen, B. 2005. Is there really more biodiversity in Mediterranean forest ecosystems? *Taxon* 54, 905–910.
- Farjon, A. 2010. *A handbook of the world's conifers*. Koninklijkr Brill NV, Leiden.
- Fletcher, W.J., Sánchez Goñi, M.F. 2008. Orbital and sub-orbital scale climate impacts on vegetation of the western Mediterranean basin over the last 48 000 years. *Q. Res.* 70, 451–464.
- Fletcher, W.J., Debret, M., Sánchez Goñi, M.F. 2013. Mid-Holocene emergence of a low-frequency millennial oscillation in western Mediterranean climate: Implications for

- past dynamics of the North Atlantic atmospheric westerlies. *The Holocene* 23, 153–166.
- Fletcher, W.J., Zielhofer, Ch. 2013. Fragility of western Mediterranean landscapes during Holocene rapid climate changes. *CATENA* 103, 16–29.
- Girod, C., Vitalis, R., Leblois, R., Fréville, H. 2011. Inferring population decline and expansion from microsatellite data: a simulation based evaluation of the Msva method. *Genetics* 188, 165–179.
- Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from <http://www.unil.ch/izea/software/fstat.htm>
- Guillot, G., Santos, F., Estoup, A. 2008. Analyzing georeferenced population genetics data with Geneland: a new algorithm to deal with null alleles and a friendly graphical user interface. *Bioinformatics* 24, 1406–1407.
- Hampe, A., Petit, R.J. 2005. Conserving biodiversity under climate change: the rear edge matters. *Ecol. Lett.* 8, 461–467.
- Hardy, O.J., Vekemans, X. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Mol. Ecol. Notes* 2, 618–620.
- Hardy, J., Charbonnel, N., Fréville, H., Heuertz, M. 2003. Microsatellite allele sizes: a simple test to assess their significance on genetic differentiation. *Genetics* 163, 1467–1482.
- Hood, G.M. 2010. PopTools version 3.2.5. Available on the internet. URL <http://www.poptools.org>
- Jaramillo-Correa, J.P., Grivet, D., Terrab, A., Kurt, Y., De-Lucas, A.I., Wahid, N., Vendramin, G.G., González-Martínez, S.C. 2010. The Strait of Gibraltar as a major biogeographic barrier in Mediterranean conifers: a comparative phylogeographic survey. *Mol. Ecol.* 19, 5452–5468.

- Juan, A., Fay, M.F., Pastor, J., Juan, R., Fernández, I., Crespo, M.B. 2012. Genetic structure and phylogeography in *Juniperus oxycedrus* subsp. *macrocarpa* around the Mediterranean and Atlantic coasts of the Iberian Peninsula, based on AFLP and plastid markers. *Eur. J. For. Res.* 131, 845–856.
- Kadereit, J.W., Arafah, R., Somogyi, G., Westberg, E. 2005. Terrestrial growth and marine dispersal? Comparative phylogeography of five coastal plant species at a European scale. *Taxon* 54, 861–876.
- Konnert, M., Bergmann, F. 1995. The geographical distribution of genetic variation of silver fir (*Abies alba*, Pinaceae) in relation to its migration history. *Plant Syst. Evol.* 196, 19–30.
- Krijgsman, W., Hilgen, F.J., Raffi, I., Wilson, D.S. 1999. Chronology, causes and progression of the Messinian salinity crisis. *Nature* 400, 652–655.
- Lande, R., Engen, S., Sæther, B.E. 2003. Stochastic population dynamics in ecology and conservation. Oxford University Press, Oxford.
- Liepelt, S., Bialozyt, R., Ziegenhagen, B. 2002. Wind-dispersed pollen mediates postglacial gene flow among refugia. *PNAS* 99, 14590–14594.
- Liepelt, S., Cheddadi, R., de Beaulieu J-L., Fady, B., Gömöry, D., Hussendörfer, E., Konnert, M., Litt, Longauer, R., Terhürne-Berson, R., Ziegenhagen, B. 2009. Postglacial range expansion and its genetic imprints in *Abies alba* (Mill.) — A synthesis from palaeobotanic and genetic data. *Rev. Palaeobot. Palynol.* 153, 139–149.
- Liepelt, S., Mayland-Quellhorst, E., Lahme, M., Ziegenhagen, B. 2010. Contrasting geographical patterns of ancient and modern genetic lineages in Mediterranean *Abies* species. *Plant. Syst. Evol.* 284, 141–151.

- Linares, C.J. 2011. Biogeography and evolution of *Abies* (Pinaceae) in the Mediterranean Basin: the roles of long-term climatic change and glacial refugia. *J. Biogeogr.* 38, 619–630.
- Liu, T.S. 1971. A monograph of the genus *Abies*. Publication of the Department of Forestry, Collage of Agriculture, National Taiwan University, Taipei.
- Lumaret, R., Mir, C., Michaud, H., Raynal, V. 2002. Phylogeographical variation of chloroplast DNA in holm oak. *Mol. Ecol.* 11, 2327–2336.
- Lumaret, R., Tryphon-Dionnet, M., Michaud, H., Sanuy, A., Ipotesi, E., Born, C., Mir, C. 2005. Phylogeographical variation of chloroplast DNA in cork oak (*Quercus suber*). *Ann. Bot.* 96, 853–861.
- Mantel, N. 1967. The detection of diseases clustering and generalized regression approach. *Cancer Res.* 27, 209–220.
- Martrat, B., Grimalt, J.O., Lopez-Martinez, C., Cacho, I., Sierro, F.J., Flores, J.A., Zahn, R., Canals, M., Curtis, J.H., Hodell, D.A. 2004. Abrupt temperature changes in the Western Mediterranean over the past 250,000 years. *Science* 306, 1762–1765.
- Médail, F., Diadema, K. 2009. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *J. Biogeogr.* 36, 1333–1345.
- Ortiz, M.A., Tremetsberger K, Talavera, S., Stuessy, T., García-Castaño, L.J. 2007. Population structure of *Hypochaeris salzmanniana* DC. (Asteraceae), an endemic species to the Atlantic coast on both sides of the Strait of Gibraltar, in relation to Quaternary sea level changes. *Mol. Ecol.* 16, 541–552.
- Palamarev, E. 1989. Paleobotanical evidences of the Tertiary history and origin of the Mediterranean sclerophyll dendroflora. *Plant Sys. Evol.* 162, 93–107.
- Parducci, L., Szmidt, A.E. 1999. PCR-RFLP analysis of cpDNA in the genus *Abies*. *Theor. Appl. Genet.* 98, 802–808.

- Parducci, L., Szmidt, A.E., Ribeiro, M.M., Drouzas, A.D. 2001. Taxonomic position and origin of the endemic Sicilian fir *Abies nebrodensis* (Lojac.) Mattei based on allozyme analysis. *For. Gen.* 8, 119–127.
- Peakall, R., Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* 6, 288–295.
- Pérez-Obiol, R., Jalut, G., Julià, R., Pélachs, A., Iriarte, J.M., Otto, T., Hernández-Beloqui, B. 2011. Mid-Holocene vegetation and climatic history of the Iberian Peninsula. *The Holocene* 21, 75–93.
- Pons, A., Reille, M. 1988. The Holocene and upper Pleistocene pollen record from Padul (Granada, Spain). A new study. *Palaeo. Palaeoclim. Palaeoeco.* 66, 243–263.
- Pyhäjärvi, T., Salmea, M.J., Savolainen, O. 2008. Colonization routes of *Pinus sylvestris* inferred from distribution of mitochondrial DNA variation. *Tree Gen. Genom.* 4, 247–254.
- Raymond, M., Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* 86, 248–249.
- Roucoux, K.H., de Abreu, L., Shackleton, N.J., Tzedakis, P.C. 2005. The response of NW Iberian vegetation to North Atlantic climate oscillations during the last 65 kyr. *Quat. Sci. Rev.* 24, 1637–1653.
- Rousset, F. 2008. Genepop'007: a complete reimplementation of the Genpop software for Windows and Linux. *Mol. Ecol. Res.* 8, 103–106.
- Sánchez Goñi, M., Cacho, I., Turon, J-L., Guiot, J., Sierro, F., Peyrouquet, J., Grimalt, J., Shackleton, N. 2002. Synchronicity between marine and terrestrial responses to millennial scale climatic variability during the last glacial period in the Mediterranean region. *Clim. Dyna.* 19, 95–105.

- Scaltsoyiannes, A., Tsaktsira, M., Drouzas, A.D. 1999. Allozyme differentiation in the Mediterranean firs (*Abies*, Pinaceae). A first comparative study with phylogenetic implications. *Plant Sys. Evol.* 216, 372–380.
- Schmidt, I., Bradtmöller, M., Kehl, M., Pastoors, M., Tefelmaier, Y., Weninger, B., Weniger, G-Ch. 2012. Rapid climate change and variability of settlement patterns in Iberia during the Late Pleistocene. *Quater. Inter.* 274, 179–204.
- Schütt, P. 1991. *Tannenarten Europas und Kleinasiens*. Basel, Boston, Berlin.
- Sękiewicz, K., Sękiewicz, M., Jasińska, A.K., Boratyńska, K., Iszkulo, G., Romo, A., Boratyński, A. 2013. Morphological diversity and structure of West Mediterranean *Abies* species. *Plant Biosys.* 147, 125–134.
- Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139, 457–462.
- Terhüne-Berson, R., Litt, T., Cheddadi, R. 2004. The spread of *Abies* through Europe since the last glacial period: combined macrofossil and pollen data. *Veg. Hist. Archaeobot.* 13, 257–268.
- Terrab, A., Talavera, S., Monserrat, A., Paun, O., Stuessy, T.F., Tremetsberger, K. 2007. Genetic diversity at chloroplast microsatellites (cpSSRs) and geographic structure in endangered West Mediterranean firs (*Abies* spp., Pinaceae). *Taxon* 56, 409–416.
- Thompson, J.D. 2004. *Plant evolution in the Mediterranean*. Oxford University Press, Oxford.
- Van Andel, T.H., Tzedakis, P.C. 1996. Paleolithic landscapes of Europe and environs, 150,000–25,000 years ago: an overview. *Quarter. Sci. Rev.* 15, 481–500.
- Vendramin, G.G., Degen, B., Petit, R.J., Anzidei, M., Madaghiele, A., Ziegenhagen, B. 1999. High level of variation at *Abies alba* chloroplast microsatellite loci in Europe. *Mol. Ecol.* 8, 1117–1126.
- Villar, L., Sesé, J.A., Ferrández, J.V. 1997. *Flora del Pirineo Aragonés*. Consejo de Protección

de la Naturaleza de Aragón, Huesca.

- Waples, R.S., Do, C. 2008. LDNe: a program for estimating effective population size from data on linkage disequilibrium. *Mol. Ecol.* 8, 753–756.
- Weir, B.S., Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38, 1358–1370.
- Werner, O., Sánchez-Gómez, P., Carrión-Vílches, M.A., Guerra, H. 2002. Evaluation of genetic diversity in *Pistacia lentiscus* L. (Anacardiaceae) from the southern Iberian Peninsula and North Africa using RAPD assay. Implications for reforestation policy. *Israel J. Plant Sci.* 50, 11–18.



Fig. 1. *Abies maroccana* population at Rif Mountains (Jbel Lakraa, Morocco).



Fig. 2. *Abies tazaotana* at Rif Mountains (Jbel Tasaot, Morocco) with the cones at the top of the trees.

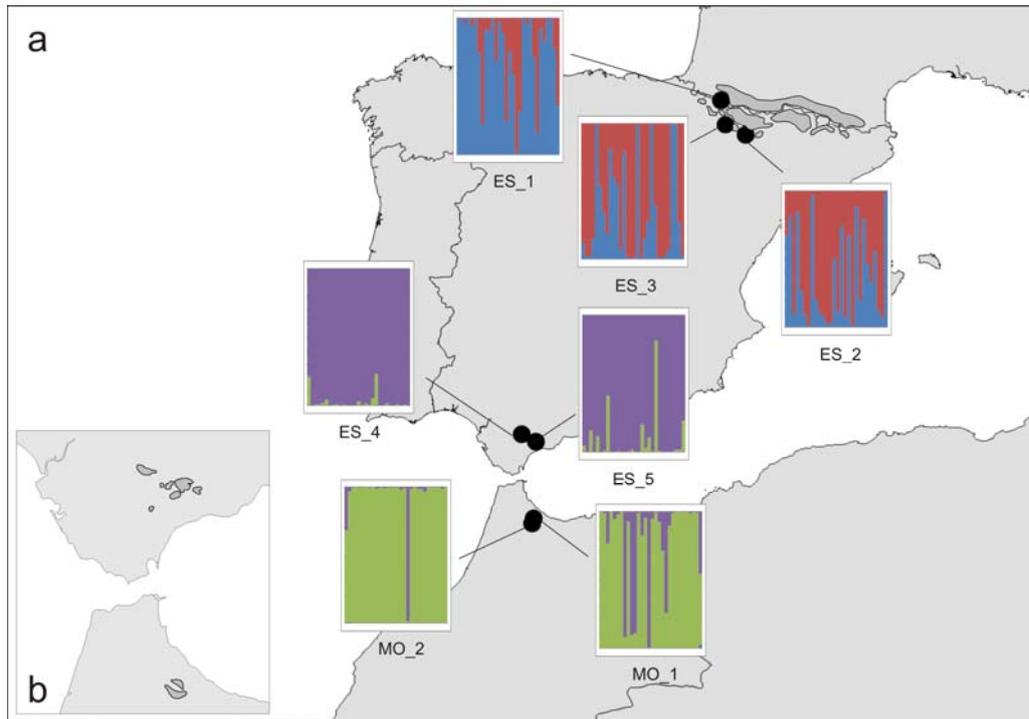


Fig. 3. a) Location of studied fir populations and their population genetic structure according to a Bayesian approach. Each vertical line denotes an individual and each of the colours denotes one of the four clusters inferred; b) Geographic range of *A. pinsapo* (Spain, Iberian Peninsula), *A. tazaotana* and *A. maroccana* (Morocco, Africa), population ID as in Table 1

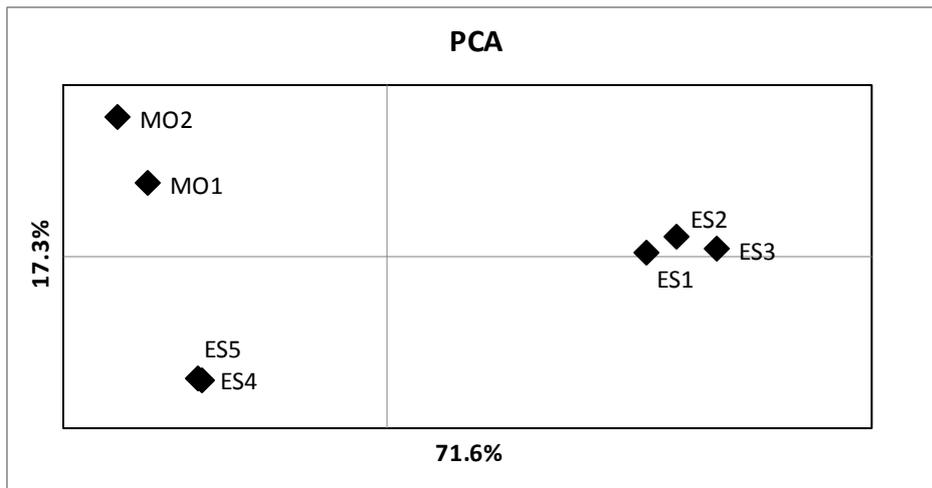


Fig. 4. Results of PCA analysis based on six nSSR markers in seven *Abies* populations, population ID as in Table 1

Table 1. Location of the studied populations

Population	ID	N	Long. [W]	Lat. [N]	Alt. [m a.s.l.]	Species
Belagua, Pyrenees, Spain	ES_1	30	00°55'	42°58'	1600	<i>A. alba</i>
La Selva, Pyrenees, Spain	ES_2	28	00°30'	42°19'	1210	<i>A. alba</i>
San Juan de la Pena, Pyrenees, Spain	ES_3	30	00°40'	42°30'	1400	<i>A. alba</i>
Sierra de Zafalagar, Spain	ES_4	29	05°22'	36°47'	1100	<i>A. pinsapo</i>
Serrania de Ronda, Spain	ES_5	30	05°03'	36°39'	1130	<i>A. pinsapo</i>
Rif Mountains, Jbel Tasaot, Morocco	MO_1	30	05°06'	35°14'	1600	<i>A. tazaotana</i>
Rif Mountains, Jbel Lakraa, Morocco	MO_2	30	05°08'	35°09'	1500	<i>A. maroccana</i>





