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Research Article

# Genetic Differentiation of *Abies equi-trojani* (Asch. & Sint. ex Boiss) Mattf. Populations from Kazdağı, Turkey and the Genetic Relationship between Turkish Firs belonging to the *Abies nordmanniana* Spach Complex

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**Abstract:** The present study aimed to test the utility of RAPD (randomly amplified polymorphic DNA) and cpSSR (simple sequence repeats) markers for in situ gene conservation programs for fir species, as well as for determining the genetic similarities between the *Abies nordmanniana* Spach species complex (*A. nordmanniana*, *A. bornmuelleriana* Matff., *A. equi-trojani* (Asch. & Sint. ex Boiss) Mattf.) and between populations of *A. equi-trojani*, which is a narrow-endemic to Turkey. For this purpose, DNA was extracted and pooled from 15 seed megagametophytes (megs) of the Ortaköy population of *A. nordmanniana* and the Muratdere population of *A. bornmuelleriana* species, and from two 7-meg subsamples each from of the Kazdağı and Çan populations of *A. equi-trojani*. Template DNA was screened with the DNA markers to reveal the amount of genetic variation in each species. It appeared that template DNA pooling for screening the fir populations with RAPD or cp-SSR markers could be effectively used to speed up gene conservation and taxonomic studies. It is suggested that DNA pooling for template DNA in the PCR (polymerase chain reaction) mixture should be limited to 7 megs, but at least 5 replications that sample different sets of families each time.

Based on genetic similarity and distance values, it seems that the Turkish fir species studied, as well as *A. equi-trojani* populations, have genetically been well differentiated. Fragmented distribution of 3 fir species in northern Turkey, belonging to the *nordmanniana* fir complex, also supports the possible existence of an ancestral species. However, to test this further studies that include fir species from the region, such as *A. cephalonica Loud., A. borisii-regis Mattf.,* and *A. cilicica* Ant. & Kotschy, are needed.

Key Words: *Abies equi-trojani*, *A. nordmanniana*, *A. bornmuelleriana*, Genetic similarity, Genetic distance, RAPD Markers, cp-SSR

## Introduction

Kazdağı, located in north-western Turkey, is ecologically and floristically diverse, containing a number of plant species endemic to Turkey. Kazdağı fir (*Abies equi-trojani* (Asch.& Sint. ex Boiss) Mattf.), a narrow endemic that naturally occurs in the Kazdağı, has been identified as a priority target plant species in a national plan, The In Situ Conservation of Plant Genetic Diversity in Turkey (Kaya et al., 1997). Because of its unique growth form and ability to grow faster than other fir species in Turkey, Kazdağı fir has great potential as a genetic resource for planting and breeding programs. Kazdağı fir is limited to a total area of about 3600 ha, with a number of small isolated populations ranging in size from 120 ha to 2400 ha at elevations of 400 m-1650 m (Figure 1A, 1B). This fir has the most limited natural distribution among the Turkish fir species belonging to the *A. nordmanniana* Spach fir complex, which includes *A. nordmanniana*, *A. bornmuelleriana* Mattf., and *A. equi-trojani* (Figure 1A).

The project entitled, In Situ Conservation of Plant Genetic Diversity in Turkey, ( referred as the In Situ Conservation Project for the remainder of this paper) was initiated in selected pilot sites in 1993 with the financial

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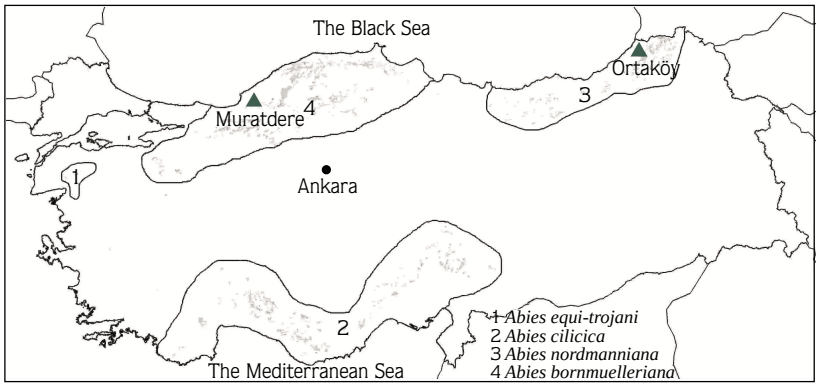
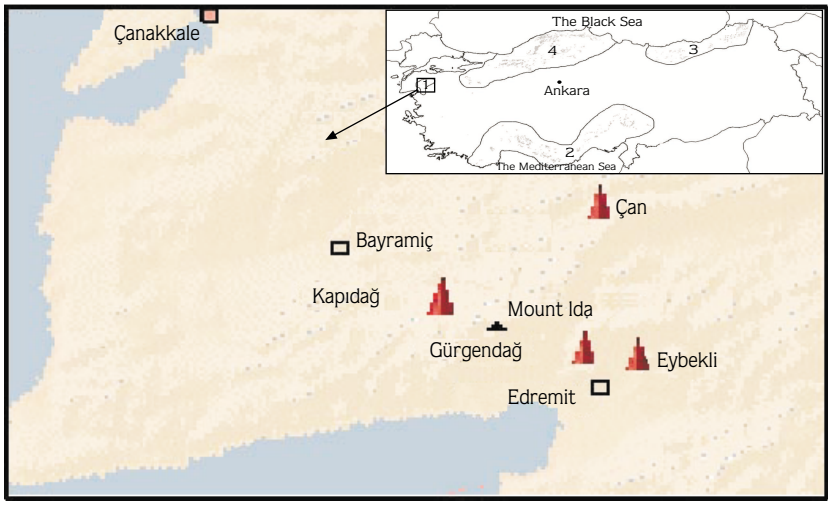



A




B

Figure 1. A. The natural range of *Abies* species in Turkey. B. The natural distribution of *Abies equi-trojani* and location of *A. equi-trojani* populations sampled for the study.

support of the World Bank, under the Global Environmental Facility (GEF) fund. As part of the In Situ Conservation Project, a national plan was prepared to implement in situ conservation of plant genetic resources nation-wide (Kaya et al., 1997). The Kazdağı fir was also identified as a priority target plant species by the national plan, which produced a list of priority species for in situ conservation (Kaya et al., 1997).

To determine the magnitude and pattern of genetic variation in natural populations of Kazdağı fir, in order to set up in situ reserves of adequate size and number (such as gene management zones), Gülbaba et al. (1996) carried out an isozyme study by sampling all possible populations in the Kazdağı area in north-western Turkey (Figure 1B). This study indicated that mean estimates of genetic diversity parameters for all sampled populations were marginally lower ($H_e$ = 0.12) than the average reported for conifers ($H_e$ = 0.15) and was consistent with averages found for other fir species ($H_e$ = 0.13). The Çan (0.14) population, which is small, isolated, and distantly located from the core population of Gürgendağ, had higher genetic diversity than the core population ($H_o$ = 0.08). Additionally, the estimated Nei's unbiased genetic distance between the isolated Kapıdağ and Çan populations was higher than that of the Çan and core populations (Gülbaba et al., 1996). There is also a dispute among taxonomists about whether the Kazdağı fir is a separate species of the taxa, *A. nordmanniana* (Ata, 1975; Farjon & Rushforth, 1989; Şimşek, 1991).

With the advance of molecular markers, it is now

possible to use DNA markers to address the problems of conservation genetics and plant taxonomy (Kaya & Neale, 1995; Vendramin et al., 1996; Parducci and Szmidt, 1999; Isoda et al., 2000; Parducci et al., 2001). There are now many DNA markers available as well as random amplified polymorphic DNA (RAPD) (Kaya and Neale, 1995) and simple sequence repeats (SSR) (Vendramin et al., 1996) markers, which can be used to answer practical and theoretical questions in forest genetics, such as seed source certification, in situ conservation of genetic resources, and the taxonomic relationship between species complexes. For instance, Parducci and Szmidt (1999) reported that based on the pattern of cpDNA variation, species occurring in adjacent regions (e.g. *Abies alba* Mill., *A. bornmuelleriana*, *A. nordmanniana*, *A. cephalonica* Loud. var. *cephalonica*, and *A. cilicica* Ant. & Kotschy) maintained the same group of haplotypes, while taxa growing in scattered and isolated areas (*A. nebrodensis* Mattei, *A. numidica* De Lannogex Carrieri, *A. pinsapo* Boiss. var. *marocana* (Trab.) Ceballos & Martin, *A. pinsapo* var. *pinsapo*, *and A. concolor* Lindl. ex Hildebr. var. *concolor*) were characterised by different haplotypes. They suggested that these 2 groups of taxa are genetically differentiated.

Thus, in the present study, seed material from *A. nordmanniana, A. bormuelleriana*, and 2 populations of *A. equi-trojani* were studied to assess genetic diversity in these species using RAPD and cp-SSR primers. Additionally, it was aimed to determine the genetic relationships between the *A. nordmanniana* species complex as well as between populations of *A. equi-trojani.*

## Materials and Methods

### Plant Materials

Bulk seeds of *Abies bornmuelleriana* from Muratdere seed stands in Bilecik province (north-western Turkey) and of *A. nordmanniana* collected from Ortaköy seed stands in Artvin province (north-eastern Turkey) were obtained from the Forest Tree Seeds and Tree Breeding Research Directorate (FTSBRD) of the Ministry of Environment and Forestry, Ankara (Table 1A, Figure 1A). Topographic and geographic information for the seed sources provided in Table 1A were obtained from the forest management plans for the forests from which seed sources originated. For *A. equi-trojani*, open pollinated family seeds of 4 populations were available from a previous collection. These seeds were collected by FTSBRD for isozyme studies (Gülbaba et al., 1996) as a part of In Situ Conservation Project activities in the Kazdağı area (Kaya et al., 1997) (Table 1B, Figure 1B). In this study, only 2 populations, Kapıdağ and Çan, were selected and used (Table 1, Figure 1). The topographic, geographic, and stand-related information about the Kapıdağ and Çan populations of *A. equi-trojani* were obtained from the forest management plans for the forests from which seed sources originated. Open pollinated seeds from 23 mother trees (families) of the Kapıdağ population (close to the core population of Gürgendağ) and from 22 families of the Çan population (the most distantly located one with respect to Gürgendağ) were obtained from FTSBRD.

Table 1. A. Locations of *Abies bornmuelleriana* and *Abies nordmanniana* seed sources.
B. Locations of *Abies equi-trojani* populations.

| A)<br>Species Name | Population | Latitude<br>(N) | Longitude<br>(E) | Elevation<br>(meter) | Area<br>(ha) | Aspect | Age |
|---|---|---|---|---|---|---|---|
| *A. bornmuelleriana* | Muratdere Seed Stand<br>(Bilecik Province) | 39°51'45" | 29°44'00" | 1650 | 114.0 | North-west | 100 |
| *A. nordmanniana* | Ortaköy Seed Stand<br>(Artvin Province) | 41°16'37" | 41°57'47" | 1600 | 203.5 | North | 52 |
| B)<br>Species Name | Population | Latitude<br>(N) | Longitude<br>(E) | Elevation<br>(meter) | Area<br>(ha) | Population<br>size (total) | Sample<br>size |
| *Abies equi-trojani* | Çan<br>Kapıdağ | 39°56'00"<br>39°43'20" | 26°07'00"<br>26°52'00" | 7500<br>1450 | 123<br>250 | 43,100<br>87,500 | 22<br>23 |

### DNA isolation procedures

Seeds were soaked in distilled water at 4 °C for 24 h and seed megagametophytes were excised from the seed coat and embryo.

For DNA extraction purposes, 15 megagametophytes were ground from *A. nordmanniana* and *A. bornmuelleriana* seed sources, while two-DNA extraction pools, each including 7 megagametophytes originating from different mother trees within each of the 2 *A. equi-trojani* populations (Kapıdağ and Çan), were prepared. DNA pooling from different genotypes within a seed source or taxonomic entity (species, subspecies, varieties, etc.) would be a useful approach for quick and cost effective screening of materials for fingerprinting and genetic relationship studies. Thus, DNA pooling with a different number of genotypes was carried out to determine the appropriate number of genotypes for DNA pooling. DNA extraction was performed according to the method of Dellaporta et al. (1983). Pooled megagametophyte tissues were homogenized with a glass pestle in 400 µl of the extraction buffer (100 mM of Tris. HCL (pH 8.0), 50 mM of EDTA, 500 mM of NaCl, and 10 mM of β-mercaptoethanol) in 2-ml Eppendorf centrifugation tubes. After complete homogenization of the megagametophyte tissues (30-40 s), an additional 400 µl of extraction buffer was added to make a final volume of 800 µl. Homogenized tissues were placed in a 60 °C water bath for 30 min. After incubation, 250 µl of 5 M potassium acetate was added, mixed, and incubated on ice for 30 min. Then, it was centrifuged at 0 °C for 15 min. DNA was extracted with 0.5 ml of phenol-chloroform (1:1) by mixing, via inversion and centrifugation for 5 min. Then, 500 µl of the top layer in the Eppendorf tube was transferred to a 1.5-ml Eppendorf centrifugation tube. Afterwards, 350 µl of isopropanol was added to the Eppendorf tubes, mixed by inversion, and centrifuged for 5 min. The pellet was rinsed with 70% refrigerated ETOH, and the extracted DNA was dissolved in 50 µl of TE buffer and stored at 4 °C for later use.

### Primers and PCR Reaction Conditions

Random 10-base oligonucleotide primers were obtained from Operon Technologies (Alameda, California, USA) and from the University of British Columbia (UBC) (Vancouver, Canada). Chloroplast SSR primers were synthesised, as described in Vendramin et al. (1996). After preliminary screening of the RAPD primers with good PCR products, a total of 113 RAPD primers (65 of them from Operon Industries, California, USA, and 48 were from UBC, Vancouver, Canada) were selected for use in the study. The list and sequence of these primers are available upon written request sent to the senior author. In addition, 20 chloroplast SSR (cp-SSR) primers were included in the study (Table 2). These primers were originally developed for pines (Vendramin et al., 1996), but it has been shown that these SSR primers could be used in fir species as well for the purpose of population genetic studies (Vendramin & Ziegenhagen, 1997).

The PCR reaction procedures and DNA amplification cycles for RAPD primers were modifications of Kaya and Neale (1995). A typical 25-µl PCR reaction volume contained 1 µl of template DNA (3 ng/µl), 1 µl of primer (Operon primers or 2 µl of UBC primers) 2.55 µl of deoxynucleoside triphosphate mix (100 mM of each nucleotide; Promega), 2.4 µl of buffer (100 mM of Tris-HCl (pH 8.3), 500 mM of KCl, Boehringer, Manheim), 0.8 units of DNA polymerase (Promega, USA), 5.0 µl of mineral oil (Sigma M-5904), and 9.89 µl or 8.89 µl of double-distilled $H_2O$, depending on whether Operon or UBC RAPD primers were used. DNA amplification was carried out with the following amplification procedure: 85 °C for 15 sec, 95 °C for 5 sec, 92 °C for 1 min and 55 sec., 95 °C for 5 sec, 92 °C for 55 sec., 37 °C for 1 min, and 72 °C for 2 min., This cycle was repeated 44 times and kept at 72 °C for 2 min.

The PCR reaction procedures and DNA amplification cycles for cp-SSR primers were modifications of Vendramin et al. (1996). A typical 25-µl PCR reaction volume contained 8 µl of template DNA (4 ng/µl), 10 µl of SSR primers (1 ng/µl), 0.16 ml of Taq DNA polymerase (1 unit), 2.4 µl of buffer (100 mM of Tris-HCl (pH 8.3), 500 mM of KCl, Boehringer, Manheim), 2.55 µl of deoxynucleoside triphosphate mix (100 mM of each nucleotide, Promega), and 1.79 µl of double-distilled $H_2O$. The DNA amplification procedures were the same as described above for RAPD primers. All amplification products were visualized on 2% agarose gel (2.4% for SSR primers) (Figure 2A, B). The gels were run in 1 × TAE buffer (0.4 M of Tris Acetate, 1 mM of EDTA) at 130 volts (or 0.2 amperes) for 3 h. Then, the gels were stained with ethidium bromide (0.5 mg/ml) for 30 min.

Table 2. The list of cp-SSR primers used in the study (Vendramin et al., 1996).

| Published locus name | Forward (F) and Reverse (R) primer sequences |
|---|---|
| Pt1254 | F)5'-CAATTGGAATGAGAACAGATAGG-3'<br>R)TGCGTTGCACTTCGTTATAG |
| Pt9383 | F)AGAATAAACTGACGTAGATGCCA<br>R)AATTTTCAATTCCTTTCTTTCTCC |
| Pt15169 | F)CTTGGATGGAATAGCAGCC<br>R)GGAAGGGCATTAAGGTCATTA |
| Pt26081 | F)CCCGTATCCAGATATACTTCCA<br>R)TGGTTTGATTCATTCGTTCAT |
| Pt30204 | F)TCATAGCGGAAGATCCTCTTT<br>R)CGGATTGATCCTAACCATACC |
| Pt36480 | F)TTTTGGCTTACAAAATAAAAGAG<br>R)AAATTCCTAAAGAAGGAAGAGCA |
| Pt41093 | F)TCCCGAAAATACTAAAAAAGCA<br>R)CTCATTGTTGAACTCATCGAGA |
| Pt45002 | F)AAGTTGGATTTTACCCAGGTG<br>R)GAACAAGAGGATTTTTTCTCATACA |
| Pt48210 | F)CGAGATTGATCCGATACCAG<br>R)GAGAGAACTCTCGAATTTTTCG |
| Pt51873 | F)AATCTTTCTACGGAACGGAAA<br>R)ATCATTTTGTGCTATGCAATGA |
| Pt63718 | F)CACAAAAGGATTTTTTTTCAGTG<br>R)CGACGTGAGTAAGAATGGTTG |
| Pt71936 | F)TTCATTGGAAATACACTAGCCC<br>R)AAAACCGTACATGAGATTCCC |
| Pt79951 | F)CTTTTGTTTTTCAACAATTGCA<br>R)ACATCTATCTCCCATATCGGC |
| Pt87268 | F)GCCAGGGAAAATCGTAGG<br>R)AGACGATTAGACATCCAACCC |
| Pt100783 | F)ATACGTATGTATCCCCTAACTGTCA<br>R)TCAATTTTTGCCATATCCTGA |
| Pt102584 | F)TTCATGTAATTCCCAGATCCA<br>R)CATTATGTGCGCGATAATTTC |
| Pt107148 | F)GTTCATTCGGGATCCTTAAAA<br>R)GTACTTTCCTTCAGCCAATCTG |
| Pt107517 | F)AAAGCTTTTATTGCGGCC<br>R)ATGGCAGTTCCAAAAAAGC |
| Pt109567 | F)TATTATCGAACAACGAGAATAATCC<br>R)TCACTGTCACTCTACAAAACCG |
| Pt110048 | F)TAAGGGGACTAGAGCAGGCTA<br>R)TTCGATATTGAACCTTGGACA |

### RAPD and cp-SSR primer data collection and evaluation

The RAPD fragments, which were amplified with high resolution and consistently produced by RAPD and SSR primers, were determined and labelled according to their length as base pairs (bp). The RAPD fragments amplified and not-amplified in each seed source were determined and a data file with presence (1) or absence (0) of the RAPD fragments (marker) was created to be used later in statistical and genetic analyses. Since template DNAs from haploid megagametophyte tissues were used, DNA fragments amplified by a given primer will be allele(s) of that locus generated by a given primer-pair because the cp-SSR markers are co-dominant markers with multiple alleles. Thus, cp-SSR data were also organized as an allele of cp-SSR locus. The SSR allele (s) of cp-SSR locus was also determined in each seed source and a data file with presence (1) or absence (0) of allele(s) of a given locus was formed to treat both RAPD fragments (marker) and SSR locus alleles in the same data set as marker data.

The number of markers (RAPD fragments and cp-SSR locus alleles) common or unique to the studied *Abies* species, as well as the populations of *A. equi-trojani*, was calculated by using the PROC MEANS and PROC FREQ procedures of the SAS Statistical Package (SAS User's Guide, 1988). The genetic distance between all pairs of species (or populations) was calculated using the following formula (Marsolais et al., 1993):

$$\text{Genetic distance } (D_{xy}) = 1 - ((n\text{XY})/(n\text{X} + n\text{Y} - n\text{XY}))$$

Where *nXY* is the number of shared markers between species *X* and *Y*, *nX* is the number of markers present in species *X*, and *nY* is the number of markers present in species *Y*. The genetic similarity indices between species were also estimated with the following formula:

$$\text{Genetic similarity } (S_{xy}) = n\text{XY}/(n\text{X} + n\text{Y} - n\text{XY})$$

*nXY, nX,* and *nY* are described the same as above.

Moreover, estimates of Nei's (1972) standard genetic identity (*I*) and standard genetic distance (*D*), unbiased for sample size (Nei, 1978) for all pair-wise seed sources, were calculated to show the genetic relationships between the studied seed sources and to compare them to the results from Marsolais et al. (1993). The standard genetic distance (*D*) of all pair-wise species comparisons was calculated to show the genetic relationships between species with the following formula:

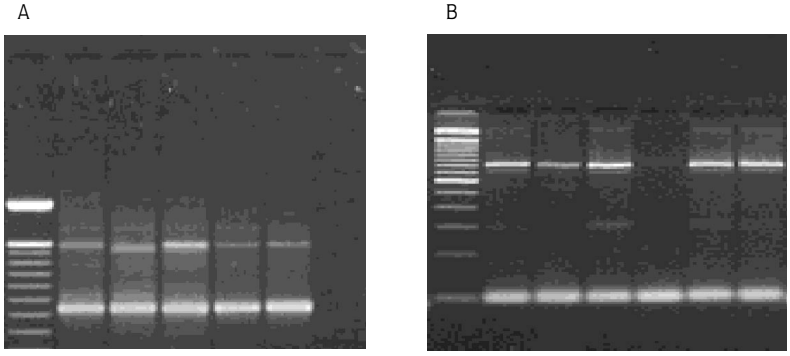



Figure 2. A. PCR product using RAPD primer F19. Lane 1: marker (100-bp pair ladder); lane 2: *Abies bornmuelleriana*; lane 3: *Abies nordmanniana*; lane 4: *A. equi-trojani*-Kapıdağ-1; Lane 5: *A. equi-trojani*-Kapidağ-2; lane 6: *A. equi-trojani*-Çan-1; lane 7: *A. equi-trojani*-Çan-2. B. PCR product with cp-SSR primer 48210. Lane 1: marker (100-bp pair ladder); lane 2: *Abies bornmuelleriana*; lane 3: *Abies nordmanniana*; lane 4: *A. equi-trojani*-Kapıdağ-1; Lane 5: *A. equi-trojani*-Kapıdağ-2; lane 6: *A. equi-trojani*-Çan-1, lane, 7: *A. equi-trojani*-Çan-2.

$$D = -Ln\ I$$

where  is the identity between 2 species (seed sources), *x* and *y*.

The genetic identity ($I_{xy}$) between 2 seed sources was calculated as:

$$I_{xy} = \Sigma X_i Y_i / \sqrt{\Sigma X_i^2 \Sigma Y_i}$$

where $X_i$ and $Y_i$ represent frequency of the *i*th marker in seed sources of X and Y.

Overall genetic identity (I) was calculated as:

$$I = J_{xy} / \sqrt{J_x * J_y}$$

where $J_x$, $J_y$, and $J_{xy}$ are the arithmetic means of all studied markers of $\Sigma X_i^2$, $\Sigma Y_i^2$, and $\Sigma X_i^2 \Sigma y_i^2$.

All estimations were made with the use of POPGENE (Yeh et al., 1999) and GeneStat computer programs (Lewis, 1993). A dendrogram was also constructed with POPGENE software using the genetic distance matrix based on Nei (1972) (Yeh et al., 1999).

## Results

### DNA markers yielded by RAPD and cp-SSR Primers, and the utility of template DNA pooling

Among the tested RAPD primers, 81.4% yielded reproducible DNA fragments (markers). The number of DNA markers obtained by RAPD primers ranged from 260 in the *A. equi-trojani*-Kapıdağ population to 294 in other seed sources, while 20 cp-SSR primers produced 34 reproducible genetic markers in all the studied Turkish fir seed sources.

Among the 20 tested cp-SSR primers, 75% produced amplified PCR products in Turkish fir species. Since cp-SSR primers were developed mainly for pine species, some of the primers produced amplified-DNA products that were out of the expected range (greater than expected, 200 bp) or did not yield any amplified DNA product in any PCR condition.

For rapid screening and cost reduction, differentiation of populations with RAPD and cp-SSR primers, and sub-sampling in the Kapıdağ population of *A. equi-trojani* by pooling template DNA from 7 different megagametophytes, each originating from different families, was also carried out. Sub-sampling within the population further improved the detection of additional RAPD fragments and cp-SSR alleles (markers) for the Kapıdağ population of *A. equi-trojani*. For example, by using one more-pooled template DNA (Kapıdağ sample 2, Table 3) 29 more markers were detected. Additionally, in each sub-sample the number of unique genetic markers ranged from 27 (Kapıdağ sample 2) to 33 (Kapıdağ sample 1) in *A. equi-trojani*. The presence of unique markers in sample 2, especially with RAPD primers, indicated that to have adequate representation of the population in sample DNA, template DNA pooling should be performed with several subsamples of the 7 megagametophytes, including different genotypes.

### Genetic similarities

There were 188 shared markers (out of 280) among *A. nordmanniana* and *A. bornmuelleriana.* The number of genetic markers unique to each species, produced either by RAPD primers or SSR primers, was almost equal; there were 44 genetic markers unique to *A. nordmanniana* and 46 genetic markers unique to *A. bornmuelleriana* (Table 3). Based on RAPD and cp-SSR primer-data, the genetic similarity of *A. nordmanniana* and *A. bornmuelleriana* was 67.62% (Table 4A). The Nei's (1972) genetic identity estimate was also in the same range (72.1%) for these species (Table 4B).

The number of markers shared by *A. equi-trojani* and other Turkish fir species was about the same. There were 194 markers (out of 270) shared by *A. equi-trojani* and *A. bornmuelleriana*, and 195 shared by *A. equi-trojani and A. nordmanniana* (Table 3). When *A. nordmanniana* and *A. bornmuelleriana* were compared to *A. equi-trojani*, with respect to unique genetic markers, there were 15 and 17 genetic markers unique to *A. nordmanniana* and *A. bornmuelleriana,* respectively. The number of genetic markers unique to *A. equi-trojani*, with respect to 2 fir species, was about the same (range: 55-59) (Table 3). The genetic similarities between *A. equi-trojani* and *A. bornmuelleriana* (71.85%) and between *A. equi-trojani* and *A. nordmanniana* (72.22%) were of similar magnitude (Table 4A)

The Nei's (1972) genetic identity estimates between *A. equi-trojani* and other Turkish fir species were of the same magnitude; ranging from 69.4% between *A. equi-trojani* and *A. bornmuelleriana* to 70.5% between *A. equi-trojani and A. nordmanniana* (Table 4B).

The number of markers shared by Kapıdağ and Çan populations of *A. equi-trojani* was 160 (out of 268). When these populations were compared, with respect to unique markers, there were 83 and 25 markers unique to Kapıdağ and Çan populations, respectively (Table 3). The genetic similarity between these populations was 59.7% (Table 4A), less than the genetic similarity index estimated between *A. nordmanniana* and *A. bornmuelleriana* species. When the Nei's (1972) genetic identity estimates between the populations and *A. equi-trojani* species were compared, the Çan population was the least similar to the species (73.1%, Table 4B).

Table 3. The number of unique and shared RAPD and cp-SSR markers of *Abies bormuelleriana*, *A. nordmanniana*, and *A. equi-trojani*.

| | RAPD | cl-SSR | Total |
|---|---|---|---|
| *Abies bornmulleriana* vs. *A. nordmanniana* (shared markers) | 163 | 25 | 188 |
| *A. bornmulleriana* (unique markers) | 41 | 3 | 44 |
| *A. nordmanniana* (unique markers) | 42 | 4 | 46 |
| Shared /Total markers | | | 188/280 |
| *A. equi-trojan*i vs. A. bornmulleriana (shared markers) | 166 | 28 | 194 |
| *A. bornmulleriana* (unique markers) | 17 | 0 | 17 |
| *A. equi-trojan*i (unique markers) | 59 | 0 | 59 |
| Shared/Total markers | | | 194/270 |
| *A. equi-trojani* vs. *A. nordmanniana* (shared bands) | 166 | 29 | 195 |
| *A. nordmanniana* (unique markers) | 15 | 0 | 15 |
| *A. equi-trojan*i (unique markers) | 55 | 5 | 60 |
| Shared/Total markers | | | 195/270 |
| *A. equi-trojani*-Kapidağ vs. Çan populations (shared markers) | 134 | 26 | 160 |
| *A. equi-trojan*i-Kapidag (unique markers) | 76 | 7 | 83 |
| *A. equi-trojan*i-Can (unique markers) | 24 | 1 | 25 |
| Shared/Total markers | | | 160/268 |
| *A. equi-trojani*-Kapidağ population samples (shared markers ) | 177 | 28 | 205 |
| Kapıdağ (Sample 1) (unique markers) | 33 | 2 | 35 |
| Kapıdağ (Sample 2) (unique markers) | 27 | 2 | 29 |
| Shared/ Total markers | | | 205/269 |

Table 4. A. Genetic similarities (Marsolais 1993). B. Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal).

| A) Codes for species | AB[1] | AN[2] | AE[3] | AE-C[4] |
|---|---|---|---|---|
| AB | – | 0.6626[a]<br>0.7810[b]<br>0.6762[c] | 0.6860<br>0.8750<br>0.7185 | |
| AN | | – | 0.7033<br>0.8500<br>0.7220 | |
| AE | | | – | |
| AE-K[5] | | | | 0.5720<br>0.7640<br>0.5970 |

B)

| Codes for Species | AB | AN | AE | AE-K | AE-C |
|---|---|---|---|---|---|
| AB | – | 0.721 | 0.705 | 0.771 | 0.558 |
| AN | 0.327 | – | 0.694 | 0.747 | 0.582 |
| AE | 0.349 | 0.366 | – | 0.914 | 0.731 |
| AE-K | 0.260 | 0.292 | 0.089 | – | 0.642 |
| AE-C | 0.584 | 0.542 | 0.314 | 0.444 | – |

[1]AB: Abies bormuelleriana; [2]AN: *Abies nordmanniana*; [3]AE: *Abies equi-trojani*; [4]AE-C: *Abies equi-trojani*-Çan; [5]AE-K: *Abies equi-trojani*-Kapıdağ. [a]Genetic similarity estimated from RAPD data, [b]genetic similarity estimated from cp-SSR data, and [c]genetic similarity estimated from combined data (RAPD and cp-SSR).

The Nei's standard genetic distance (D), unbiased for sample size (Nei, 1978), calculated for all pair-wise seed sources ranged from 8.9% between *A. equi-trojani* and the *A. equi-trojani*-Kapıdağ population to 58.4% between the *A. equi-trojani*-Çan population and *A. bornmulleriana* (Table 4B). The dendrogram based on Nei's (1978) genetic distance indicates that genetically, *A. equi-trojani* and A. *bornmulleriana* were most similar groups, followed by *A. nordmanniana.* Interestingly, the *A. equi-trojani*-Çan population was genetically the most distant seed source among the studied Turkish fir species (Figure 3).

## Discussion

The use of RAPD and cp-SSR primers in combination generated a large number of genetic markers that improved the characterisation of the Turkish fir species seed sources used in the study. It appears that RAPD markers were more sensitive to PCR conditions compared to the cp-SSR markers and required careful checking to determine if they were true amplifications. On the other hand, markers (alleles) generated by cp-SSR primers and base pair-differences between samples of less than 4-5 base pairs (bp) were not possible due to under-detection, and data on additional markers was lost. In the future, to distinguish differences between bands less than 4 bp (in order to include more allelic data in analysis for establishing better evolutionary relationships between seed sources) sequence analysis of amplified PCR products should be taken into consideration.

The sampling procedure is important for maximising the utility of markers. Seeds from *A. nordmanniana* and *A. bornmulleriana* seed sources were bulk samples collected from seed stands. In order to have better representation of the species, a family structured sampling of seed sources and several seed sources should

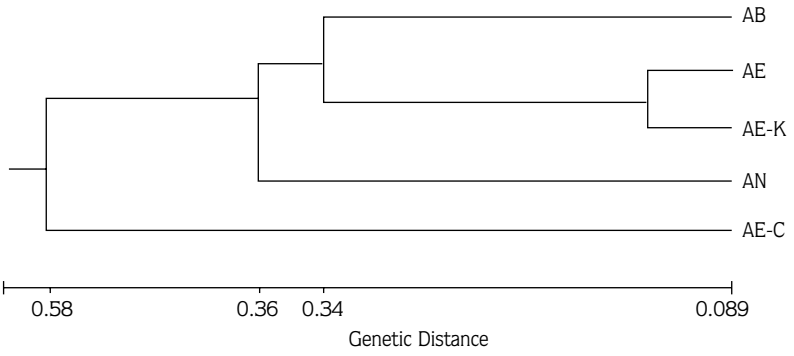



Figure 3. Dendrogram based on Nei's (1978) genetic distance. Method: UPGMA, modification of the NEIGHBOR procedure of PHYLIP v.3.5. AB: *Abies bornmuelleriana*; AN: *Abies nordmanniana*; AE: *Abies equi-trojani*; AE-C: *Abies equi-trojani*-Çan; AE-K: *Abies equi-trojani*-Kapıdağ.

be included. For rapid screening and cost reduction purposes, bulking of template DNA from 7 seeds (preferable coming from 7 different genotypes) appeared to work for finger printing and species characterisation, as it was used for quantitative loci mapping studies (Wang & Paterson, 1994). Although 5-6 template DNA bulks (each having DNA from 7 genotypes) per seed source are sufficient for detecting the existing genetic diversity of a given seed source, the effective number of megagametophytes in bulking should be determined in an iterative way by adding one more megagametophyte each time to the bulked extraction procedure. The procedure should be repeated until a maximum possible number of megagametophytes is reached. This maximum number will provide the same information as when all the megagametophytes are separately analysed.

Although the present study had limited sampling from *A. nordmanniana* and *A. bornmuelleriana* species, the preliminary results with a large number of markers yielded by RAPD and cp-SSR primers suggest that 3 Turkish fir species (*A. nordmanniana*, *A. bornmuelleriana*, and A. *equi-trojani*) may have evolved from an ancestral species, such as the *nordmanniana*, which in the past may have had continuous distribution from the Caucasus Mountains to Kazdağı. The current fragmented distribution of 3 fir species in northern Turkey, belonging to the *nordmanniana* fir complex, also supports the possible existence of an ancestral species, which could have been fragmented by intensive past human activities in the region. However, this needs to be tested with a thorough sampling including other species, such as *A. cephalonica* from Greece, *A. borisii-regis* from Bulgaria, and *A. cilicica* from southern Turkey.

From the number of shared bands, and Nei's (1972) genetic identity estimates between *A. equi-trojani* and other fir species, it appears that *A. equi-trojani* shares genetic background with *A. bornmuelleriana* and *A. nordmanniana* of similar magnitude. Nevertheless, genetic distances among species indicated that *A. equi-trojani* is closer to *A. bornmuelleriana* than to *A. nordmanniana*, following the geographic proximity of the species (Figure 3). Furthermore, the genetic distance between *A. bornmuelleriana* and *A. nordmanniana* is less than the genetic distance between *A. equi-trojani* and other fir species. When populations of *A. equi-trojani* were compared to *A. bornmuelleriana* and *A. nordmanniana*, as well as to *A. equi-trojani*, at the species level, the Çan population appeared to be genetically different from the other populations of fir species. For example, the genetic distance between the Çan population and the studied fir species ranged from 0.31 (between Çan and *A. equi-trojani*) to 0.58 (between Çan population and *Abies bornmuelleriana*; 0.54 between Çan Population and *Abies nordmanniana*). However, the previous isozyme study by Gülbaba et al. (1998) reported that there was no deficiency in genetic diversity at the species level compared to conifers in general, and no significant differences between observed and expected genetic diversity in the isolated populations, such as Çan. Additionally, the results of a common garden study dealing with adaptive seedling traits of *A. equi-trojani* (Çiçek et al., 2005) showed that in general, the studied

populations were not clearly distinguishable from each other, but only the Çan population was found to be relatively distant. Small population size and isolation from core populations do not seem to have any effect on the magnitude of genetic diversity in the Çan population. By considering that the natural range of narrow-endemic species of Kazdağı fir is restricted to a 3600-ha area, the existence of high genetic variation in adaptive seedling traits could be explained by the occurrence of substantial gene flow between isolated and core populations of the species, but this needs to be tested since the current data were not suitable for estimating the gene flow parameter for the seed sources. Also, further studies investigating genetic and evolutionary relationships among Turkish firs using family-structured sampling would be useful. Future studies should also determine the effective population size of the isolated Çan population and gene flow pattern among *A. equi-trojani* populations.

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