Differences among *Juniperus excelsa* populations as revealed at morphological traits

Abstract: The comparison of phenotypic trait differentiation and genetic differentiation at selectively neutral genetic markers can indicate divergent selection on traits. Phenotypic trait differentiation ($P_{ST}$) and two multivariate analysis methods were used to determine the level of differentiation and relationships among seven *Juniperus excelsa* populations based on 13 morphological characters of their cones, seeds, juvenile seedlings and 1+0 year old seedlings. Significant differences among populations were found for all morphological characters ($P<0.001$) apart from cotyledon length using ANOVA. According to Penrose and Squared Euclidean distances, the southeastern populations Bucak-Kestel and Gölhisar-Gölhisar (0.970; 12.374) were most similar. Aksu-Sorgun and Eğirdir-Barlapopulations, separated by a mountain range, were the most different populations (4.647; 47.157). Evaluated as a whole, both multivariate analysis methods gave similar results. Phenotypic trait differentiation ($P_{ST}$) for 1+0 year old seedlings that were grown in a common environment was similar in magnitude for the majority of traits as genetic differentiation at nuclear microsatellite markers ($F_{ST}$) suggesting the absence of divergent selection on these traits.

Additional key words: Hierarchical cluster analysis, Penrose distance, Phenotypic trait differentiation

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Introduction

Not all variation observed in nature is hereditary, instead part of the variation can be due to environmental effects (Eriksson and Ekberg 2001). Thus, the expression of morphological characters of plant species in their natural habitat is affected to different degrees by genetic and environmental factors (Mal and Lovett-Doust 2005). Measurement, description and analysis of morphological variation are fundamental steps to answer questions of biological adaptability (Ge and Hong 1995). Morphological variation within plant species growing in different habitats could suggest environmental effects or indicate genetic differences among populations. The establishment of common garden experiments including different provenances grown under common environmental conditions can help us to separate genetic from environmental effects and to understand the manner, mechanism and influencing factors of plant adaptation and evolution (Yang 1991).

Moreover, the results from morphological variation studies have provided an estimate of relatedness among populations within species (Gezer et al. 2006). Genetic variation underlying phenotypic traits and phenotypic plasticity are particularly important when the long-term stability of forest ecosystems is increasingly threatened by environmental stresses and mismanagement. Thus, a genetic characterization of natural forest resources is an essential step for
a better understanding of genetic resources for the implementation of in-situ and ex-situ conservation activities (Turna et al. 2001). In this context, the characterization of local genetic resources is often based on the knowledge of variation in morphological characters (Delgado et al. 2001).

Juniperus excelsa Bieb. M. is one of the major species for afforestations as it is resistant to drought, frost damages and poor soils growing at the boundaries of steppe in the interior parts of the mountains in its distribution areas (Saatçioğlu 1969; Browicz 1982; Yücedağ et al. 2010; Douaihy et al. 2011). In Turkey, it has a wide natural distribution in northern, western, central, and southern Anatolia, especially in the Taurus and Anti-Taurus Mountains (Yaštırk 1993) and grows between altitudes of 300 to 2300 m (Çolak and Rotherham 2007). This species, forming pure and mixed stands in Turkey (Avşar and Tonguç 2003), has economical functions due to the durability of its timber (Uçar and Balaban 2002), and organic and inorganic components in their cones (Baytop 1999). Besides, it plays an important role in afforestation programs on eroded soils and in landscaping (Gültekin 2007).

Multivariate analyses of morphological characters have proved to be suitable in assessing variation within populations and can discriminate different population types. For example, these kinds of studies have been conducted in Cedrus libani A. Rich. (Yahyaoğlu et al. 2001), Pinus sylvestris L. (Ayan et al. 2005; Şevik et al. 2010) and Juniperus excelsa Bieb. M. (Barbero et al. 1994; Mazur et al. 2004; Marcysiak et al. 2007; Douaihy et al. 2012). Besides, one study of Crimean juniper estimated the amount of genetic variation based on morphological characteristics within and among populations and heritability for a limited number of cone, seed and juvenile seedling characteristics (Yücedağ et al. 2010), and two studies applied genetic markers to assess genetic diversity within and among populations (Douaihy et al. 2011, Douaihy et al. 2012; Yücedağ and Gailing 2013).

In the present study we apply multivariate analyses to compare J. excelsa populations from Turkey based on a comprehensive set of 13 cone, seed, juvenile and 1+0 year-old seedling characters and compare phenotypic trait differentiation with differentiation at microsatellite markers (Yücedağ and Gailing 2013). Up to now only in one study (Douaihy et al. 2012) levels of differentiation among populations at phenotypic characters and genetic markers have been compared for Juniperus excelsa.

The purpose of the present study was (1) to determine morphological dissimilarities among seven populations of Crimean juniper in Lakes District of Turkey based on cone, seed and seedling characters, and (2) to evaluate to what extent phenotypic trait differentiation ($P_{ST}$) estimated in this study would resemble the genetic differentiation ($F_{ST}$) described on the basis of genetic markers reported in an earlier study (Yücedağ and Gailing 2013). Higher phenotypic differentiation than differentiation at genetic markers ($F_{ST}$) would be indicative of divergent selection on these traits. We expect that phenotypic differences among one-year-old seedlings from different populations grown in a common environment reflect genetic differences, while environmental factors have a stronger effect on phenotypic differences among populations for young seedling and cone characteristics.

Materials and methods

Plant Material

Cones and seeds were collected from 70 open-pollinated parent trees of seven populations sampled from the Lakes District in Turkey during November of 2006 (Fig. 1). Coarse and healthy cones and sound seeds were used to measure their characters [CD – cone diameter (mm); NSC – the number of seeds per cone; NSSC – number of sound seeds per cone; TSW – thousand seed weight (g)] (Yücedağ et al. 2010). For these measurements, 180 cones randomly selected for each population were used.

Measurement Procedures

Seeds to which a pretreatment procedure had been applied (Yücedağ et al. 2010) were sown in polyethylene tubes with 13 × 25 cm dimensions in Eğirdir Forest Nursery (45 km northeast of Isparta, at 920 m a.s.l.) in February of 2007. As a growing medium, a mixture containing 50% of silt and forest soil was used. The sowing depth was 2 mm. The layout of the experiment was a randomized complete block design with three repetitions. Cultivation activities such as irrigation and weed control were regularly performed.

Fig. 1. Location of Juniperus excelsa populations used in the study
for the experimental area during the growing season of 2008.

CL – Cotyledon length (cm), HL – Hypocotyl length (cm), EL – Epicotyl length (cm) and RL – Radicular length (cm) of 300 juvenile seedlings per population that were 75 days old, and SH – shoot length (cm) and RCD – root collar diameter (mm), LRL – the longest root length (cm), SFW and RFW – fresh weight of stem and root (g) of 150 one-year-old seedlings randomly selected from each population were measured.

**Statistic Treatment**

For multivariate analyses, variables measured at different scales were standardized to equally contribute to the analysis (Yahyaoglu et al. 2001). The symmetry and unimodality of frequency distributions of measured character values were verified, to assess the suitability of the data for subsequent statistical analyses (Zar 1999).

The Penrose distance method, considering data of mean and variance, was used to calculate multivariate distances among pairs of populations. This method takes into account within population variation by weighting each variable by the inverse of its variance, but does not account for correlations among variables (Yahyaoglu et al. 2001; Manly 2005). A distance close to zero in this method is the most similar to the mean vector. In addition, this distance method has an important advantage over other distance measures since it allows comparing results across studies. Thus it is frequently used to estimate genetic structure, establish forest gene maps and compare population pairs at phenotypic traits (Yahyaoglu et al. 2001). We also performed Mantel tests (999 permutations) in GENALEX 6.41 (Peakall and Smouse 2006) to check for significant correlations between morphological and geographical distances among pairs of populations.

Hierarchical cluster analyses, completely based on numerical analysis, are aiming to group objects based on their similarity through different steps to determine consecutive clusters and the distance values (or similarities) of the units to be included in these clusters. In these analyses, the clusters are not known in advance (Özdamar 2004). An ANOVA was performed to determine whether or not the populations were all equal. In this one-way variance analysis, populations were considered as fixed variables. Furthermore, the mean values and standard deviations for each of the seven populations based on the 13 characters were calculated. Morphological differences among populations were visualized with both an unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Penrose distances and hierarchical cluster analyses based on Squared Euclidean distances, using the computer program PHYLIP v3.63 (Felsenstein 1989). Calculations for Penrose distance, ANOVA and hierarchical cluster analyses were performed by using a spreadsheet and SPSS program (SPSS Inc. 2011).

Analogous to Quantitative Trait Differentiation (QTD; Spitzke 1993) among population phenotypic variance was estimated for all characters as $P_{ST} = \sigma^2_{G(among)}/(\sigma^2_{G(among)}+2\sigma^2_{G(within)})$, with $\sigma^2_{G(among)}$ being the variance among populations and $\sigma^2_{G(within)}$ the variance within populations (Raeymaekers et al. 2007, Pujol et al. 2008). Additionally, phenotypic trait differentiation ($P_{ST}$) among populations estimated in the present study was compared with genetic differentiation ($F_{ST}$) at selectively neutral microsatellite markers for the same populations published before (Yücedağ and Gailing 2013).

**Results**

Mean values of cone, seed, juvenile and 1 + 0 year old seedling characters, their standard deviations, ranges, F ratios and significance levels are given in Table 1. The analysis of variance showed significant differences between populations at the 0.001 probability level for all morphological characters with the exception of cotyledon length ($P = 0.002$). Cone diameter (CD) contributed a significant portion to the differentiation among populations ($F_{CD} = 15.25; P < 0.001$). While differences in the cone, seed and juvenile seedling characters among populations were affected by the parental environments in the natural populations, young (1 + 0 year old) seedling characters were assessed in a common garden experiment minimizing the differences among populations that are due to environmental effects. Differentiation at phenotypic traits ($P_{ST}$) ranged from 16% to 57% for cone and seed characters, from 0.4% to 19% for juvenile seedling characters and from 1% to 5% for 1 + 0 year old seedlings (Table 1). Population Eğirdir-Balkan (P6) showed the lowest performance for the majority of the morphological characters.

Morphological and Squared Euclidean distances between population pairs are given in Table 2. According to these distance values, southwestern populations Buca-Kestel (P1) and Gölhisar-Gölhisar (P4) were the most similar (P1,3 = 0.970; 12.374), whereas the northern populations Aksu-Sorgun (P2) and Eğirdir-Barla (P4) were the most different (P2,4 = 4.647; 47.157). Even though Eğirdir-Y. gökdere (P5), it was geographically close to Eğirdir-Balkan (P6), it showed a higher similarity (P5,7 = 1.906; 22.422) to Eğirdir-Barla (P6) with weak phylogeographic patterns could be detected. Thus, the southwestern populations Gölhisar-Göl—
Table 1. Mean ± standard deviation, F ratio and significance (P) for the studied characters

<table>
<thead>
<tr>
<th>Pop. Name</th>
<th>Cone and Seed Characters</th>
<th>Juvenile Seedling Characters</th>
<th>1+0 year old Seedlings Characters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD (mm)</td>
<td>NSC</td>
<td>NSSC</td>
</tr>
<tr>
<td>Bucak-Kestel</td>
<td>10.11 ±0.94</td>
<td>5.77 ±1.44</td>
<td>1.00 ±0.32</td>
</tr>
<tr>
<td>Aksu-Sorgun</td>
<td>10.11 ±1.13</td>
<td>5.02 ±1.47</td>
<td>0.79 ±0.33</td>
</tr>
<tr>
<td>Gölhisar-Gölhisar</td>
<td>8.98 ±1.03</td>
<td>5.88 ±1.66</td>
<td>1.14 ±0.40</td>
</tr>
<tr>
<td>Sütçüler-Tota</td>
<td>8.30 ±0.91</td>
<td>4.69 ±1.34</td>
<td>0.65 ±0.31</td>
</tr>
<tr>
<td>Eğirdir-Balkın</td>
<td>10.11 ±0.91</td>
<td>5.43 ±1.58</td>
<td>0.37 ±0.22</td>
</tr>
<tr>
<td>Eğirdir-Barla</td>
<td>9.54 ±1.00</td>
<td>5.56 ±1.57</td>
<td>1.31 ±0.39</td>
</tr>
<tr>
<td>Eğirdir-Y gökdere</td>
<td>9.31 ±1.04</td>
<td>4.86 ±1.52</td>
<td>0.55 ±0.29</td>
</tr>
<tr>
<td>Mean</td>
<td>9.49 ±1.18</td>
<td>5.32 ±1.57</td>
<td>0.83 ±0.34</td>
</tr>
<tr>
<td>Range</td>
<td>8.30–10.11</td>
<td>4.69–5.88</td>
<td>0.37–1.31</td>
</tr>
<tr>
<td>F ratio</td>
<td>15.25</td>
<td>4.87</td>
<td>3.67</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>P&lt;sub&gt;st&lt;/sub&gt;</td>
<td>0.32</td>
<td>0.16</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Differences among Juniperus excelsa populations as revealed at morphological traits

hisar (P3) and Bucak-Kestel (P1), and the southeastern populations Eğirdir-Barla (P6) and Eğirdir-Balkırı (P5) populations, the second group consisted of Sütçüler-Tota (P4) and Eğirdir-Y. gökdere (P7) populations. Aksu-Sorgun (P2) population individually formed a minor group.

In order to visualize the degree of similarity among populations, a cluster analysis (UPGMA) based on Penrose distances and a hierarchical cluster analysis on the basis of all measured characters was conducted (Fig. 2–3). The topology of the dendrograms did not change even if cone and seed characteristics were excluded from the analysis. According to the hierarchical cluster analysis (Fig. 2), three different groups could be distinguished at the 15.0 distance unit. The first group included Bucak-Kestel (P1), Gölhisar-Gölhisar (P3), Eğirdir-Barla (P6) and Eğirdir-Balkırı (P5) populations, the second group consisted of Sütçüler-Tota (P4) and Eğirdir-Y. gökdere (P7) populations. Aksu-Sorgun (P2) population individually formed a minor group.

In the UPGMA dendrogram (Fig. 3), the two Eğirdir populations (P6, P7) grouped together in the same cluster while they clustered in different groups in the hierarchical cluster analysis. For other populations, both Penrose distances and cluster analysis yielded similar results. Bucak-Kestel (P1) and Gölhisar-Gölhisar (P3) populations were most similar to each other and grouped in the same subgroups (Fig. 2–3). Accordingly, Aksu-Sorgun (P2) and Eğirdir-Barla (P6) populations were the most different from each other and grouped in different subgroups according to both analysis methods.

Table 2. Values (\(P_{i,j}\)) of Penrose (lower triangular matrix) and Squared Euclidean (upper triangular matrix) distances

<table>
<thead>
<tr>
<th>Pop. No</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.509</td>
<td>–</td>
<td>29.463</td>
<td>22.959</td>
<td>34.223</td>
<td>47.157</td>
<td>22.780</td>
</tr>
<tr>
<td>3</td>
<td>0.970</td>
<td>2.193</td>
<td>–</td>
<td>29.590</td>
<td>25.196</td>
<td>16.431</td>
<td>22.052</td>
</tr>
<tr>
<td>4</td>
<td>1.491</td>
<td>1.867</td>
<td>2.286</td>
<td>–</td>
<td>36.705</td>
<td>36.641</td>
<td>20.579</td>
</tr>
<tr>
<td>5</td>
<td>1.709</td>
<td>2.775</td>
<td>2.038</td>
<td>2.789</td>
<td>–</td>
<td>20.907</td>
<td>32.641</td>
</tr>
<tr>
<td>6</td>
<td>2.814</td>
<td>4.647</td>
<td>2.099</td>
<td>3.508</td>
<td>2.087</td>
<td>–</td>
<td>22.422</td>
</tr>
<tr>
<td>7</td>
<td>3.151</td>
<td>2.958</td>
<td>2.515</td>
<td>2.528</td>
<td>3.369</td>
<td>1.906</td>
<td>–</td>
</tr>
</tbody>
</table>

Fig. 2. Dendrogram of hierarchical cluster analysis based on Squared Euclidean distances among populations

Fig. 3. Cluster diagram (UPMGA) based on Penrose distances among populations
Discussion

Relatively high $P_{ST}$ values for most cone and seed characters and for juvenile seedling characters could be due to environmental effects on character expressions resulting in an overestimation of phenotypic variation among populations. $P_{ST}$ values for one-year old-seedlings (n = 150 for each population), that have been grown in a common environment, were considerably lower and in the range of genetic differentiation values ($F_{ST}$ values) observed at putatively neutral nuclear microsatellite markers ($F_{ST} = 2.8\%$) (Yücedağ and Gailing 2013). Similar differentiation at nuclear microsatellites and phenotypic traits suggests that these traits are neutral with respect to selection (e.g. Conner and Hartl 2004; Pujol et al. 2008; Whitlock 2008).

The results of the present study on cone and seed morphological characters were within the range reported by previous studies (Yaltun 1993; Gültekin 2007; Eliçin 1977; Ayaz 1980; Avşar 2004) apart from thousand seed weight that was found to be higher compared to values reported by Eliçin (1977) and Gültekin (2007) as it was determined by taking into consideration empty and sound seeds (Yücedağ et al. 2010).

When the mean values of all characters were considered (Table 1), Sütçüler-Tota population ($P_s$) revealed the highest performance in accordance with results by Gülçü and Gültekin (2005) who compared the seedling characters of five Juniperus excelsa origins from Turkey including Eğirdir-Barla and Sütçüler-Tota populations that were also analyzed in the present study. On the other hand, even though mean root collar diameter showed coherency with values observed by Avşar and Tonguç (2003) and Gülçü and Gültekin (2005), mean shoot length was higher than in the latter studies possibly due to the fact that seedlings in the present study were grown in polyethylene tubes and not in seedbeds.

The non-significant relation between morphological and geographical distances can to due to topographical barriers that affected gene flow between neighboring populations. Likewise, Turna et al. (2006) reported that it was hard to see apparent relationships between the genetic distances of populations and their geographic locations based on results of cluster analyses. Also, no significant correlation was found between geographic distances and genetic distances at nuclear microsatellite markers for five of these populations (Yücedağ and Gailing 2013).

Conclusion

All characters studied were important to determine morphological distance among populations and to group populations. Namely, the populations were not homogeneous with regard to all characters. The lack of resolution in both dendograms reflects a lack of morphological differentiation at the population level even though the overall differentiation was highly significant. Since Sütçüler-Tota population ($P_s$) revealed the highest performance compared to all other populations for the majority of characters, this population would have priority to be included in a gene conservation program. Based on just the phenotypic characters, Bucak-Kestel and Göllhisar-Göllhisar could be considered as one seed zone, since both populations are very similar morphologically. However, based on allele frequency differences at nuclear microsatellites most populations are differentiated from each other with exception of population pair Sorgun/Beyşehir (not included in the morphological analysis) (Yücedağ and Gailing 2013).

Further studies should be carried out to provide deeper insights into the amount of genetic variation in ecologically important traits that is present in these populations. For instance, traits related to drought tolerance such as water use efficiency, rate of photosynthesis and stomatal conductance of seedlings grown in common garden experiments should be assessed, and phenotypic and genetic differentiation should be determined based on the analysis of additional molecular markers and phenotypic traits. These studies would be one avenue to compare differentiation at quantitative traits and at genetic markers to identify traits that are under divergent selection. Moreover, future projects should include additional populations representing different morphological types and altitudinal and geographical variation.

Acknowledgements

We would like to express our great appreciation to Forest Engineer Emre ORTA for the contribution to measurements and also thank two anonymous reviewers for their helpful comments on the manuscript.

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