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Two new morphotypes of *Pinus eldarica*: Discrimination by macromorphological and anatomical traits

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Abstract: *Pinus eldarica* has been introduced to Iran from more than 800 years ago. Some individuals of this pine have altered both in shape and growth rate in northeastern of Iran, and generated two distinct morphotypes which are called Conical-shaped and Ball-shaped pines. This study was conducted to discriminate these morphotypes using macromorphological and anatomical characteristics. Results of macromorphological analysis showed significant differences both with univariate and multivariate analysis and consequently two new morphotypes were clearly separated from Mondell pine individuals. Furthermore, anatomical differences observed in Conical-shaped pine in comparison with two other pines, from some valuable taxonomical point of view traits such as cross-section form of needle, number of stomata per area, number and position of resin ducts etc. Furthermore, the existent difference in traits like cuticle thickness, stomata density, needle perimeter and length, state increasing the adaptation potential to aridity in Conical-shaped pine in comparison with two others. The differences of two new morphotypes demonstrate that they are new variants of Mondell pine and it is need to be used molecular markers and phylogenic studies for specifying the cause of these morphological and anatomical differences.

Additional key words: Mondell pine, Anomalous morphotypes, Anatomy, Statistical analysis

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Introduction

Mondell Pine (*Pinus eldarica* Medw.) is a drought tolerant pine with a narrow range of distribution, confined to a semi desert environment southeast of Tbilisi, Georgia, Transcaucasia. This species grows on the eastern extremity of Choban-Dagh Range, along the south side of the Iori River on individual mountain of Eller Oukhi in the confine of Azerbaidjan Republic and Georgia. It is considered to be an Oligocene relict that previously had occupied a larger area (Harrington et al. 1989, Kaundun et al. 1997, Mirov 1967).

The systematic position of *P. eldarica* has not yet been made unequivocally clear; in fact in some researchers' opinion it is a geographic variety of *P. brutia*, while according to others is a separate species (Calamassi et al. 1988). However, somewhat more than 800 years ago, this species was introduced to Iran and cultivated in the northeastern, eastern and central regions of the country. Crown shape, growth rate, cone size and may other morphological features of some individuals of Mondell pine have been altered over time and two distinct forms have been generated from the original individuals. The smaller variant has a lower growth rate and is called the Ball-shaped pine, corresponding to its shape of crown, and the other variant bears middle stature and is known as the conical-shaped pine, referring to its conical crown shape. Here we refer to the variants by these names.

The variations in plant structure that are commonly affected by genetic and environmental factors are particularly strongly expressed in the morphology and anatomy of leaves (Dickison 2000, Rudall 2007). Multivariate statistical methods, such as principal component and cluster analyses are very useful for summarizing and describing the variation found either in natural or breeding populations, especially if based on morphological data showing continuous distributions (Camussi et al. 1985, Johnson and Wichern 1992).

Plant anatomy remains highly relevant to systematic, paleobotany, and the relatively new science of developmental genetics, which interfaces disciplines and utilizes a combination of techniques to examine gene expression in growing tissues (Rudall 2007, Dickison 2000). It also is important to make the distinction between general (diagnostic) characters that enable one taxon to be separated or distinguished from another or that may imply phenetic relationship among taxis, and those characters that can be used phylogenetically (Rudall 2007, Dickison 2000). Therefore, anatomical information can be taxonomically useful without having obvious evolutionary or phylogenetic interpretation (Dickison 2000). The present study was conducted to compare and discriminate Mondell pine from its two generated morphotypes in both quantitative and qualitative aspects of morphological and anatomical structure using univariate and multivariate statistical analysis.

Methods

Plant collections and growth

Three year old seedlings of Mondell pine along with its Conical-shaped and Ball-shaped morphotypes were collected from Nashtifan (March of 2006), in eastern Iran (34°25'50'' N and 60°9'58'' E at 865 m a.s.l.), where the great morphological changes in Mondell pines were first observed. All samples collected from this location. The dwarfish morphotypes are able to be increased by sexual reproduction, thus villagers currently grow their miniature seeds (and cone) up for selling (Fig. 1).

Since each of these three pine groups showed high homogeneity, we randomly selected twenty one seedlings of these pines (Mondell, Ball-shaped and Conical-shaped pine) from the surgery of the study area. All sampled seedlings were transferred to the greenhouse in pots and grown for six months in similar situation.

Morphometric analysis

After the six months, we measured the following morphological parameters of seedlings: crown diameter, height, basal stem diameter, needle length, fascicle sheath length, and needle diameter with sheath. All needle attributes were measured on more than 70 random samples from each seedling were determined. Because the parental individuals growing in the same conditions were not numerous in the studied area, we had a few parental individuals in similar condition for collecting cones (seven individuals from

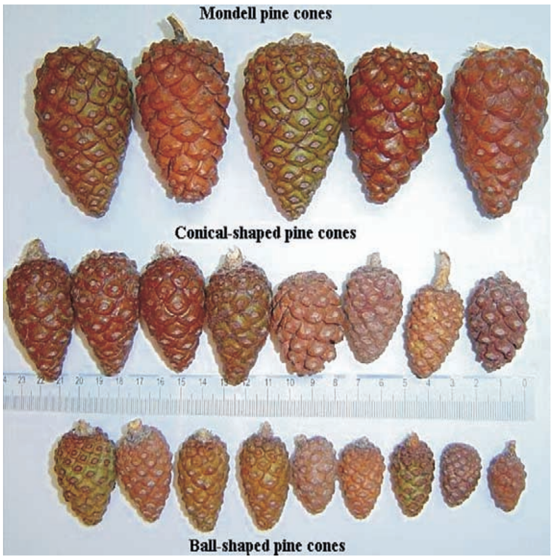

Fig. 1. Left: Some individuals of Mondell pine in row (background) and a Ball-shaped pine with similar age (14 years old trees) in front of them. Right: Sample cones of Mondell pine (up), Conical-shaped pine (middle) and Ball-shaped pine (down) collected from parental trees in study area

each pine). We measured cone and seed characters from parental individuals (results obtained from 50 cones of each individuals and their seeds), including the width of closed cones, cone length and weigh, peduncle diameter at the base of the cones, seed number per cone, seed size (without wing) and seed weight.

Anatomical study

Anatomical samples were collected from seedlings and immediately placed in a FAA solution (containing 5% Formalin, 5% Acetic acid and 90% Alcohol). Fixed materials then were transverse sections of needle and prepared by hand cutting. Sections were cleared with sodium hypochlorite, dehydrated and colored with methyl green and carmine-vest and mounted in glycerin. Afterwards, each cross-section was digitally photographed joined with a light microscope (Olympus BH2-RFCA, Japan) with different magnification. Images were transported to Adobe Photoshop CS4 Extended software for measuring the anatomical traits.

SEM Microscopy

After air-drying needle samples of three-year-old seedlings (July 2006) and mounting on aluminum stubs, 0.5 cm in diameter pieces were coated with gold film in a sputter coater (SCD 005, BAL-TEC Corporation, Switzerland), and were observed by a scanning electron microscope (XL30, Philips, Netherlands) operated at 25 kV (Barthlott et al. 1998; Tomaszewski 2004).

Statistical analyses

Finally, morphological and anatomical traits were analyzed using multivariate and univariate statistical methods such as principal component analysis, k-means cluster analysis, two step cluster, ANOVA and then Tukey HSD. All statistical analyses were tested by means of SPSS and PC-ORD software.

Results

Both univariate and multivariate analyses showed significant differences (P<0.01) between the three groups of studied pines for more than 11 morphological characters. ANOVA results for all 13 morphometric traits are shown in Table 1.

Tukey-HSD test were used to determine specific differences among 3 types of mentioned pines based on mean comparison of 13 morphological traits (Fig. 2, A-M).

Two step cluster analysis with the characters' means of parental, Ball-shaped and Conical-shaped pines revealed 2 separate clusters: one composed of Ball-shaped and Conical-shaped pines, and the other of Mondell pine, (parental) individuals. A mean comparison of 13 morphological characters of each cluster is presented in Table 2.

By running a K-means cluster analysis, morphological distances among the three pines were distinguished. In this method, each pine is represented by a cluster center. Final cluster centers of all morphological traits and the distance between final cluster centers in three types of pines are specified in Tables 3 and 4.

We used principle component analysis to determine the most useful morphological traits for specifying the maximum difference among mentioned pines. The initial total variance extracted for the first 3 axes of PCA which is presented in Table 5.

After repeating initial component analysis, we found 2 variables (morphological traits) unsuitable for this analysis based on their low measure of sampling adequacy. Therefore, the other stage was performed with 11 residual traits. Then two components with

Table 1. Analysis of variance results for 13 morphological traits of Mondell pine and its two morphotypes

Number of traits	Morphological traits	df	Mean square	Sig.	F
1	Needle diameter (with sheath)	2	0.81	0.000	38.930**
2	Sheath length	2	26.1	0.000	25.843**
3	Cone length	2	914.7	0.000	66.682**
4	Cone weight	2	1294.1	0.006	26.303**
5	Cone maximum diameter	2	481.6	0.000	76.315**
6	Cone peduncle diameter	2	26.6	0.000	40.932**
7	Number of seed per cone	2	4252.3	0.000	83.642**
8	Seed length	2	19.8	0.000	92.541**
9	Seed weight	2	0.011	0.000	141.505**
10	Seedling height	2	21562.8	0.000	169.231**
11	Seedling crown diameter	2	477.04	0.000	43.986**
12	Stem diameter	2	4.042	0.834	0.183 ns
13	Needle diameter	2	0.165	0.000	7.946**

Note: the signs of ** and ns show statistical difference in 99% significant level and no significant difference respectively.

eigenvalue >1 and either percentage of variance >10% were extracted from the others. Afterwards the model was conducted with the first 2 components which determined over 70% of total variance. The correlation between morphological traits (after eliminating stem diameter and needle sheath length, the two traits which were unsuitable for this analysis) and each 2 extracted components is presented in Table 6.

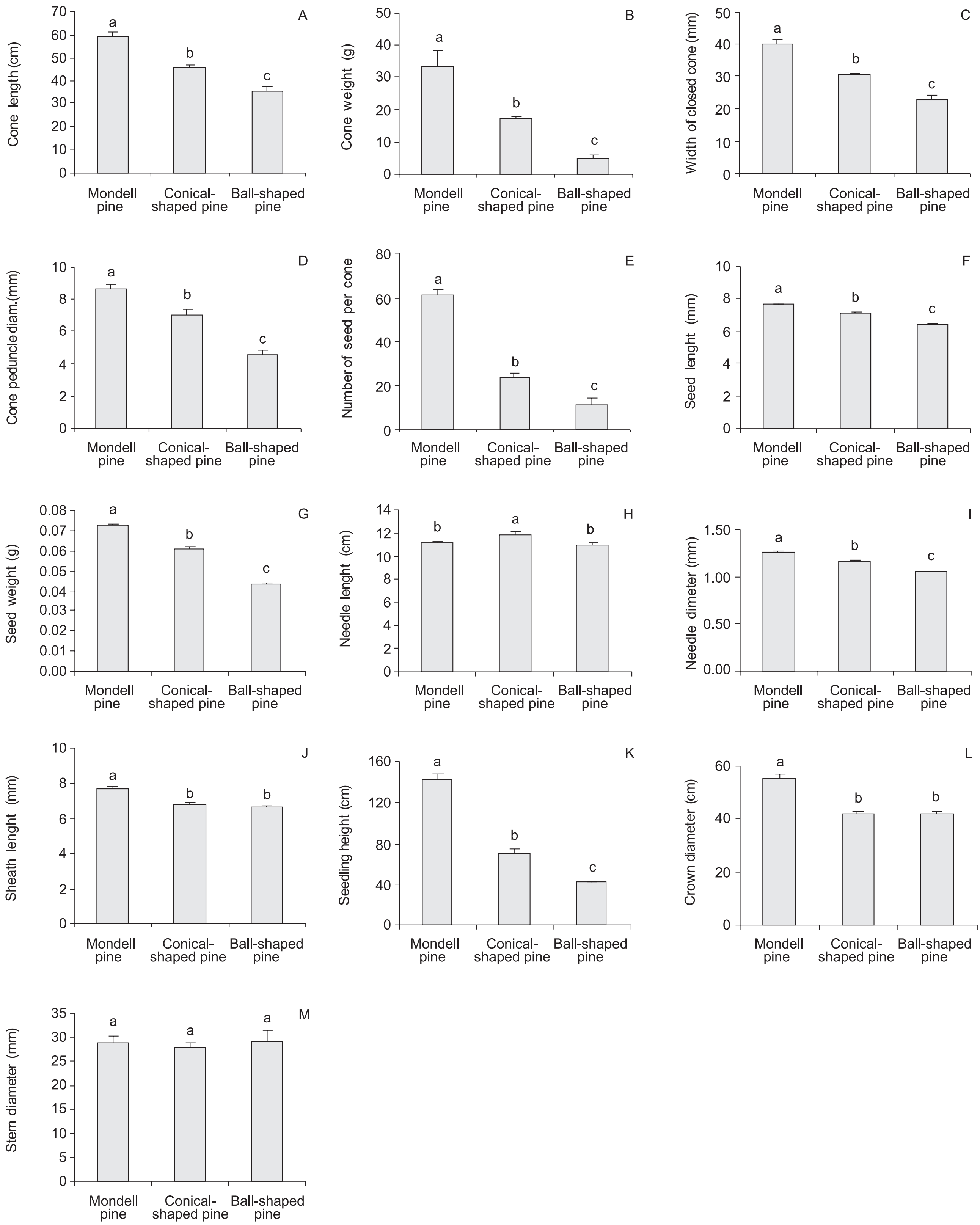

Fig. 2. Results of mean comparison of 13 morphological characters among three pines with Tukey-HSD test (Mean ± SE)

Table 2. Comparison of morphometric traits in each cluster of two step cluster analysis (Mean ± S.E)

Morphological traits	Cluster 1	Cluster 2	Sig.	t statistic
Needle diameter (with sheath)	1.11 ± 0.01 b	1.25 ± 0.02 a	0.000	–6.445**
Sheath length	6.73 ± 0.07 b	7.25 ± 1.66 a	0.000	–6.414**
Cone length	40.77 ± 1.74 b	59.21 ± 0.13 a	0.000	–6.362**
Cone weight	11.04 ± 1.77 b	33.19 ± 5.14 a	0.006	–4.074**
Width of closed cone	26.87 ± 1.22 b	40.27 ± 1.10 a	0.000	–6.694**
Cone peduncle diameter	5.80 ± 0.39 b	8.62 ± 0.30 a	0.000	–4.415**
Number of seed per cone	17.36 ± 2.59 b	90.83 ± 2.61 a	0.000	–9.996**
Seed length	6.97 ± 0.05 b	7.67 ± 0.05 a	0.000	–10.012**
Seed weight	0.057 ± 0.001 b	0.073 ± 0.001 a	0.000	–10.852**
Seedling height	56.56 ± 4.30 b	142.88 ± 5.40 a	0.000	–12.001**
Seedling crown diameter	42.13 ± 0.65 b	55.50 ± 1.51 a	0.000	–9.600**
Stem diameter	28.50 ± 0.17 a	28.81 ± 1.59 a	0.878	–0.156 ns
Needle length	11.44 ± 0.13 a	11.09 ± 0.19 a	0.106	–1.621 ns

Note: the signs of ** and ns show statistical difference between 2 separated claster in 99% significant level and no significant difference respectively.

Table 3. Final cluster centers of morphological traits among each three pines

Morphological traits	Clusters		
	Mondell pine	Ball-shaped pine	Conical--shaped pine
Needle diameter (with sheath)	1.36	1.07	1.16
Sheath length	6.92	5.88	6.52
Cone length	59.21	35.41	46.13
Cone weight	33.19	4.91	17.18
Width of closed cone	40.27	23.01	30.56
Cone peduncle diameter	8.62	4.61	6.99
Seed number per cone	60.83	11.00	23.71
Seed length	7.83	6.63	7.55
Seed weight	.0742	.0396	.0644
Seedling height	144.67	42.29	68.57
Seedling crown diameter	56.83	43.00	42.00
Stem diameter	28.00	29.64	27.93
Needle length	11.82	9.40	12.10

Ultimately, hierarchically relationships among the mentioned pines are shown by a dendrogram from Cluster Analysis by Ward's method (Fig. 4).

Analysis of variance results of all 13 anatomical needle traits of Mondell, Ball-shaped and Conical-shaped pine have been presented in Table 7.

Table 4. Morphological distances between final cluster centers of mentioned pines

Clusters	Mondell pine	Ball-shaped pine
Mondell pine		121.853
Ball-shaped pine	121.853	
Conical-shaped pine	88.957	34.541

According to Figure 4, needle form in cross-section of Mondell and Ball-shaped pine is semicircle and of Conical-shaped pine is angular. The most thick needle was related to Ball-shaped pine and the most thin was to Mondell pine ($P<0.05$).

Hypodermis is formed from three layers and occasionally one layer of oval cells with relatively thick wall and larger dimension than epidermis cells (Fig. 5). Mesophyll in these pines is formed of green uniform tissue of cells with folded wall. There are 2 resin ducts in needle cross-section of Mondell and Ball-shaped pine which may be both external and one of them be medial. While just one external resin duct exist in Conical-shaped needles and some of needles are without resin duct (Fig. 5).

Stomata exist on ventral and dorsal face of needle and are completely sunk in epidermis. Since there were more stomata on ventral face of needle because of having greater surface, we analyzed and compared the stomatal traits in ventral face. Results showed the

Table 5. Total Variance extracted in first 3 axes of Principal Components Analysis

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total
1	6.282	57.109	57.109	6.282	57.109	57.109	6.192
2	1.527	13.886	70.995	1.527	13.886	70.995	1.859
3	.958	8.705	79.700				

Table 6. Correlation between morphometric characters with each 2 extracted components

Morphological traits	Component	
	1	2
Cone diameter	.977	
Cone length	.968	
Seed number per cone	.966	
Cone weight	.926	
Cone peduncle diameter	.904	
Seedling height	.901	
Seedling crown diameter	.806	
Seed length		.736
Seed weight		.726
Needle diameter		.539
Needle length		–

Note: Two components with eigenvalue more than 1 extracted.

most density of stomata on ventral face of Ball-shaped pine (mean 81 stomata/mm^2) but the least was belong to Conical-shaped pine needles (mean 59 stomata/mm^2). Distance between stomata per row also was measured on SEM images (Fig. 6). Results showed the maximum distance between the stomata in Conical-shaped pine (P<0.01).

Discussion

Morphological analysis has been applied successfully for demonstrating the variation among such pines species as *P. nigra*, *P. brutia*, *P. taeda* as well as among many other populations of tree species (Aguinagalde et al. 1997, Aguinagalde and Bueno 1994, Chen et al. 2004, Gibson and Hamrick 1991, Isik 1986, Kara et al. 1997). Previous studies have shown that *Pinus brutia* has strong genetic variation and significant differences in growth characters associated with elevation (Isik 1986, Isik and Isik 1999, Kara et al. 1997).

The leaf in fact, has often been considered the most anatomically variable organ of the plants and could be a valuable tool in identification of provenance, hybrids and species (Calamassi et al. 1988, Dickison 2000, Salazar 1983, Snyder and Hamaker 1978). In addition to an important role in phylogenetic analysis, anatomical data can be applied toward the independent resolution in separating species, natural hybridity and other taxonomic problems as helping to place systematically difficult taxa, evaluating the taxonomic homogeneity and naturalness of taxa (Snyder and Hamaker 1978, Gallis et al. 1998, Dickison 2000, Rudall 2007).

The taxonomic records indicate that *P. brutia* in addition to its variants *P. eldarica*, *P. pithysua* etc., are extremely variable species which exhibit considerable variation both in form and growth characters in their natural range (Mirov 1967, Palmberg 1975). In this study, the results of different methods showed similar tendencies for the morphological status of the mentioned pines. The average values obtained from the 13 measured parameters used in the morphometric characterization of Mondell, Ball, and Cone-shaped pines showed statistically significant differences (P<0.01) among the three pine groups except for the stem diameter of the seedlings. Mondell pine had greater mean values of 11 characters than Conical-shaped and Ball-shaped pine (Fig. 2). We separated two distinct groups with cluster analysis. One group was composed of the two new morphotypes and the other consisted of Mondell pine (Fig. 3). The minimum morphologic distance was obtained between Ball-shaped and Conical-shaped pines and maximum distance observed between Ball-shaped and Mondell pine (Table 4 and Fig. 3). In other word Ball-shaped and Conical-shaped pines are more closely related to each other than they are to Mondell pine, and also Ball-shaped pines have diverged the farthest from Mondell pine.

Based on component analysis, cone and seed related traits allow the best basis for discrimination of the new morphotypes of *Pinus eldarica* when using univariate and multivariate analysis. In the PCA analysis, cone

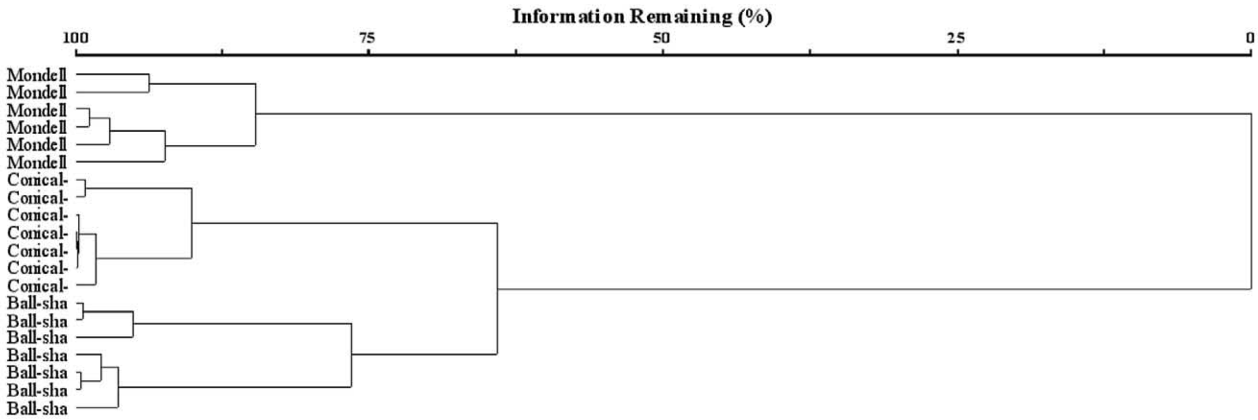

Fig. 3. Dendrogram and cluster distance measures between individuals of three mentioned pines by Ward's method

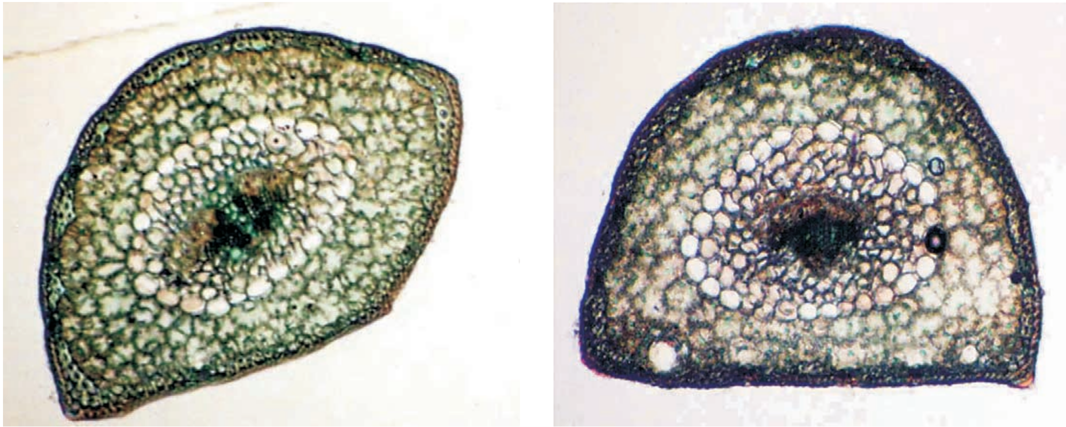

Fig. 4. Needle form in cross-section. Left: Typical form of needle in Conical-shaped pine. Right: Mondell and Ball-shaped pine (Magnification: 40×)

length, diameter and weight, cone peduncle diameter, number of seeds per cone, seedling height and crown diameter, and seed length and weight were considered as good diagnostic characters for discrimination between the Ball-shaped and Conical-shaped pines from parental individuals of Mondell pine and they were the best for distinguishing between Ball-shaped and Cone-shaped pines, and showed the highest correlation coefficient in each of the two components (Table 6). However, stem diameter and needle sheath length were not suitable characters in this analysis, because they do not distinctly explains the decreasing trends of mean values from Mondell to Conical-shaped to Ball-shaped pines, respectively. These results are in agreement with univariate analysis (Fig. 2).

In fact a few references are also available on the comparative anatomy of the resin ducts (Wu and Hu 1997). However, the number of surrounding cells of resin ducts was more in Mondell pine than two other pines. The most diameter of resin duct also was observed in Mondell pine and the least was related to Conical-shaped pine. As mentioned before there is often one resin duct in needle of Conical-shaped pine. These results may be related to the amount of resin secretion in these pines.

The number of stomata per mm^2 in ventral face of needle also is the least in Conical-shaped pine and the most in Ball-shaped pine. Moreover distance between stomata per row which is influenced of stomata density factor was the most in Conical-shaped pine. In fact, this demonstrates the less density of stomata in the morphotype.

The thickness of cuticle layer also in needle surface of Conical-shaped pine was more than the others. The current 3 characteristics (number of stomata per area, distance between stomata per row and thickness of cuticle) demonstrate the drought tolerance potential in Conical-shaped morphotype in contrast with two others.

Fewer perimeter and length of needles in Conical-shaped pine in comparison with two others, emphasis an increase of adaptation potential relative to drought due to surface exposed to light has been reduced in this morphotype. The less frequency and density of stomata and the more thickness of cuticle is an adaptation mechanism of higher plants against

Table 7. Anatomical characteristics comparison among Mondell pine and its two morphotypes (Duncan test, Mean ± Standard Error)*

Anatomical traits	Mondell pine	Conical-shaped pine	Ball-shaped pine
Number of cells around resin ducts	0.27 a ± 11.43	0.12 c ± 6.01	0.33 b ± 8.46
Number of endodermis cell	0.33 b ± 29.67	0.88 a ± 33.66	0.88 b ± 30.67
Needle diameter (mm)	0.07 b ± 1.08	0.06 ab ± 1.26	0.06 a ± 1.38
Needle cross-section height (mm)	0.04 a ± 1.20	0.02 b ± 0.89	0.07 b ± 1.00
Cross-sectional perimeter (mm)	0.04 ab ± 3.53	0.14 b ± 3.25	0.23 a ± 4.05
Stomatal density on ventral face (No./mm^2)	1.86 b ± 69.78	2.14 c ± 58.96	0.95 a ± 81.22
Distance between stomata in row of ventral face (μm)	3.14 b ± 88.05	5.10 a ± 101.91	2.31 b ± 85.78
Resin Duct diameter (μm)	2.4 a ± 72.2	3. 7 b ± 43.0	2.7 b ± 50.40
Cuticle thickness (μm)	0.21 b ± 2.16	0.37 a ± 3.84	0.29 ab ± 2.95
Hypodermis thickness (μm)	0.93 a ± 17.82	0.81 a ± 19.14	0.68 a ± 20.25
Mesophyll thickness (μm)	18 a ± 255	26 a ± 233	12 a ± 233

Various latin words in each row show significant difference, and similar words show non-significant difference in 95% significant level.*

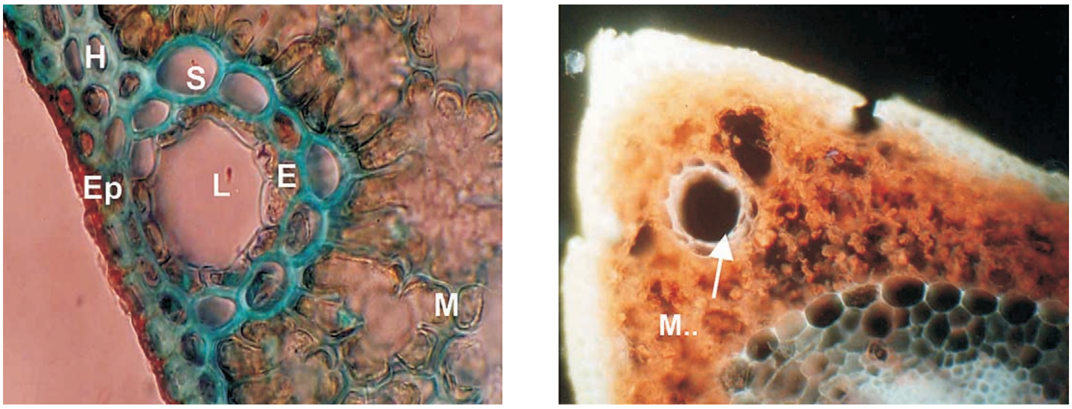

Fig. 5. Position of resin ducts. Left: external resin duct in needle of Conical-shaped pine (L: Lumen, E: Epithelial cells with thin wall, S: Sheath cells with thick wall, H: Hypodermis, Ep: Epidermis and M: Mesophyll tissue). Right: Medial resin duct (M.R.D.) in needle of Mondell and Ball-shaped pine (Magnification: 400×)

to drought conditions, because the main function of cuticle and stomata is decreasing the light radiation and controlling evaporation, transpiration and water loss to atmosphere respectively. Cuticle layer, protect photosynthesis tissues of plants against harmful and damaging radiations (Bird and Gray 2003, Matas et al. 2003, Müller and Riederer 2005, Salazar 1983).

Needle cross-section also in Conical-shaped pine has distinguished than two other pines. Based on the Figure 4 semicircle form of needle section in Mondell pine has been altered to angular form in Conical-shaped pine. Moreover, different position of resin ducts is considerable as compared with two other pines. Based on Biswas and Johri (1997) classification, in this morphotype only external duct was observed in contrast to Mondell and Ball-shaped pine which have medial dusts as well. In conifers, the resin ducts are a common structure of the plant body with important role in distinguishing and characterising the species particularly in pines, (Wu and Hu 1997, Sheue et al. 2003, Boratyńska and Bobowicz 2001). Stomatal study has been useful for discrimination of pines hybrids (Kormutak et al. 1993). The number and position of resin duct in needles may considerably and interspecifically vary, but the position is more important in taxonomic and systematic studies (Sheue et al. 2003). However, the relative position of the ducts in the needle may be used as an aid in identification (Sheue et al. 2003).

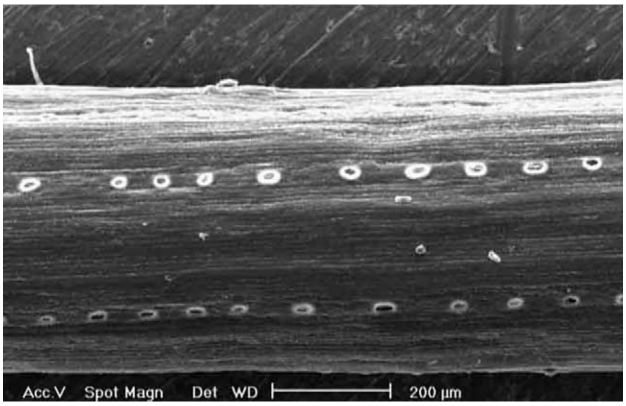

Fig. 6. Scanning Electron Microscope (SEM) from outer surface of Conical-Shaped pine: Stomata on ventral face of needle

We can conclude based on current anatomical differences in companion with the needle length and also diameter and (cross-sectional) height of needle was outer characters exposed to surrounding environ. While internal structure of needle such as hypodermis and mesophyll thickness had no significant differences (P>0.05). According to previous study, stomatal traits (stomata density) are useful for distinguishing pine hybrids (Snyder and Hamaker 1978, Matziris 1984).

Variations in needle anatomy have influence on needle physiology (Pachepsky et al. 1995). As a result, anatomical data often have proven most reliable in the refutation of claims of close relationship, rather than positive assertions of the relationship of taxa (Dickison 2000). Because our sample pines were collected from only one geographical location with uniform soil, precipitation, temperature, light, and humidity, then it can be suggested that observed differences in their morphology and anatomy are relevant for differences in their genetic profile.

Anyhow, *Pinus eldarica* introduced to Iran from many centuries ago and exposed to some morphological and physiological changes which resulted in creation of two new variants of this pine from many years before, because the oldest individual of Ball-shaped pine is more than 200 years old now. The new generated pines seem to be mutant forms of Mondell pine which can regenerate from sexual reproduction. However based on the results of this study, the use of DNA markers, cytogenetic investigations, which will likely be useful in determining the probable incidence of polyploidy, as well as analyses of the enzymes which contribute to the synthesis and polymerization of fatty acids for wax (e.g., esterase) based on recent study (Shayanmehr et al. 2008) may be important in further investigations.

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