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The evolutionary divergence of *Pinus nigra* subsp. *pallasiana* and its varieties based on noncoding *trn* regions of chloroplast genome

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Abstract: The Anatolian black pine [*Pinus nigra* Arnold subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*] is one of the subspecies of European black pine, growing naturally as a widespread mid-elevation species in the southern, western, and northern Anatolian Mountains of Turkey. Three well-recognized varieties [var. *pallasiana*, var. *fastigiata* Businsky with pyramidal form, and var. *seneriana* (Saatçioğlu) Yalt. with globular-shaped crown with multiple stems] occur naturally, but the studies on them are very limited. These 3 varieties of Anatolian black pine were sampled in natural stands where they coexist to determine evolutionary divergence in the species as well as its evolutionary relationships with other related pine species. A total of 71 trees were sampled to assess molecular divergence patterns in the species by using 3 noncoding *trn* regions (*trn*L, *trn*L-F, and *trn*V) of chloroplast DNA. The results indicated that *trn*L and *trn*V regions were conserved among Anatolian black pine taxa, but the *trn*L-F region revealed 3 parsimony-informative sites. The sequence diversity in the *trn*L-F region was useful to separate both var. *seneriana* and var. *fastigiata* from Anatolian black pine as well as this species from the other members of section *Pinus* L. The genetic divergence between 2 varieties (0.0018–0.0027) was greater than the divergence between varieties and Anatolian black pine (0.0009–0.0018). The nucleotide sequences of *trn* regions obtained from seed megagametophytes of both Anatolian black pine and var. *seneriana* stands from the same location did not show any divergence, while the *trn* sequences from needle tissues of var. *seneriana* diverged from the *trn* sequences of Anatolian black pine. This divergence suggests that var. *seneriana* may have evolved as a result of a point mutation in a gene with pleiotropic effects involved in apical dominancy of Anatolian black pine, but this has to be explored further with future studies.

Key words: Anatolian black pine, *Pinus nigra*, chloroplast DNA, genetic divergence, molecular phylogeny, *trn*

1. Introduction

Pinus nigra J.F.Arnold (European black pine) has a natural distribution in southern Europe to Turkey. The Anatolian black pine [*Pinus nigra* J.F.Arnold subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*] is one of the subspecies of European black pine, growing naturally as a widespread mid-elevation species in the Taurus and the western and northern Anatolian Mountains of Turkey (Kaya and Temerit, 1993) (Figure 1). From here on, *Pinus nigra* subsp. *pallasiana* var. *pallasiana* will be referred to as Anatolian black pine or, for short, var. *pallasiana*. Four varieties of Anatolian black pine were reported by Yücel (2000) and Boydak (2001): var. *seneriana* (Saatçioğlu) Yalt, var. *pyramidata* Hort., var. *yaltirikiana*, and var. *columnaris-pendula* Boydak. However, in the 2012 Checklist of the Flora of Turkey (Vascular Plants) (Güner et al., 2012), 2 subspecies [subsp. *nigra* and subsp. *pallasiana* (Lamb.) Holmboe] and 3 varieties [var. *seneriana* (Saatçioğlu) Yalt., var. *fastigiata* Businsky, and var. *pallasiana*] were recognized. Moreover, *P. nigra* subsp. *pallasiana* var. *pyramidata* was reevaluated as a synonym for var. *fastigiata*. Among these, only var. *seneriana* has widespread distribution in Turkey. It has a globular crown with multiple stems. It is naturally found mainly in the Bolu (Çaydurt), Manisa (Alaşehir), and Kütahya (Tavşanlı) provinces of Turkey as small groups (Ünaldı, 2005) or as individuals in all-natural Anatolian black pine forests. Meanwhile, var. *fastigiata* has a pyramidal shape with multiple stems with a limited natural distribution in Kütahya Province (Yücel, 2000). Generally, both of these varieties are clonally propagated for ornamental purposes.

There are numerous studies dealing with the magnitude and pattern of genetic variation in natural populations of Anatolian black pine (Kaya and Neale, 1993; Doğan et al., 1998; Çengel et al., 2000, 2012). These studies indicated the existence of high genetic diversity within populations. The unpublished data on Anatolian black pine and its varieties (var. *seneriana* and var. *fastigiata*) using random amplified

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polymorphic DNA markers with limited sampling of seeds indicated that these 2 varieties are genetically close to each other and are separated clearly from *P. nigra* subsp. *pallasiana* populations. In general, the cone (viable seed) productions in both these varieties are rare events. Even if trees have produced cones, most of the seeds are either empty or are very low in viability. The trees from these varieties that produce cones with seeds are most likely putative hybrids with the *Pinus nigra* subsp. *pallasiana* var. *pallasiana* trees that dominate natural stands. Thus, the evolutionary relationship between Anatolian black pine and its varieties needs to be further explored to understand the molecular basis of this divergence. The sequence diversity in chloroplast DNA (cpDNA) and nuclear *ITS* regions has been used extensively to infer phylogenies of a number of angiosperms and gymnosperms including pines (Wang et al., 1999; Lopez et al., 2002; Gernandt et al., 2003; Gil et al., 2011; Dündar et al., 2013; Yücedağ and Gailing, 2013). Furthermore, the *trn* region with Group I intron in cpDNA (Shinozaki et al., 1986; Palmer, 1991) has a conserved secondary structure (Taberlet et al., 2007) with alternation of conserved and variable regions (Quandt et al., 2004). Since noncoding sequences tend to evolve faster than coding sequences, they may provide more informative characters for phylogeny reconstruction in *Pinus* L.(Wang et al., 1999). Considering that the chloroplast genome is paternally inherited in pines (Wagner et al., 1987; Neale and Sederoff, 1989), a comparative study on conserved *trn* regions of chloroplast genomes among Anatolian black pine taxa could provide useful information for addressing the question of evolutionary divergence among them.

With the current study, we aimed to provide information on the molecular basis of evolutionary divergence of Anatolian black pine and its varieties as well as their evolutionary relationships with other related pine species with respect to sequence diversity in *trn* regions of cpDNA. This study is the first dealing with the evolutionary divergence in the species.

2. Materials and methods

2.1. Plant materials

The needle tissues and seeds of Anatolian black pine and var. *seneriana* were sampled from a Gerede-Çaydurt mixed stand (Table 1; Figure 1). Additionally, seeds were collected from the Anatolian black pine stand located in Mengen-Daren. The seeds from Gerede-Çaydurt and Mengen-Daren stands were half-sibs and were collected from parent trees with the following criteria: 1) trees had to be separated by at least 50–100 m within each stand, 2) the elevation range of parent trees had to be within 300 m, and 3) cones had to be collected from the upper one-third part of the crown of each tree to minimize inbred material. Needles were also sampled from trees by applying the first and the second criteria mentioned above.

Needle and seed samplings of var. *fastigiata* were carried out in a seed orchard established clonally by selecting pyramidal trees from the Tavşanlı-İkizoluk natural stand (Table 1; Figure 1).

2.2. DNA extraction and quantification procedures

DNA extractions were performed as described by Kaya and Neale (1993, 1995) for seed megagametophytes and Doyle

Table 1. Description of studied seed sources of Anatolian black pine and its varieties.

Seed source	Latitude	Longitude	Type of stand	Forest management directorate	Type of tissue used	Number of trees	Number of sequences
P. nigra subsp. *pallasiana* var. *pallasiana* (PNP-G-N)	40°47′N	32°12′E	Natural stand	Gerede-Çaydurt (Bolu[b])	Needle	16	16
P. nigra subsp. *pallasiana* var. *seneriana* (PNPS-G-N)	40°47′N	32°12′E	Natural stand	Gerede-Çaydurt (Bolu[b])	Needle	14	14
P. nigra subsp. *pallasiana* var. *seneriana* (PNPS-G-S)	40°47′N	32°12′E	Natural stand	Gerede-Çaydurt (Bolu[b])	Seed megagametophytes	7	7
P. nigra subsp. *pallasiana* var. *pallasiana* (PNP-M-S)	40°57′N	32°17′E	Seed stand	Mengen-Daren (Bolu[b])	Seed megagametophytes	17	17
P. nigra subsp. *pallasiana* var. *fastigiata* (PNPF-T-S)	39°27′N	29°41′E	Seed orchard[a]	Tavşanlı-İkizoluk (Kütahya[b])	Seed megagametophytes	9	9
P. nigra subsp. *pallasiana* var. *fastigiata* (PNPF-T-N)	39°27′N	29°41′E	Seed orchard[a]	Tavşanlı-İkizoluk (Kütahya[b])	Needle	8	8

[a]Seed orchard was established clonally from Tavşanlı-İkizoluk natural stand in Eskişehir Forest Nursery (40°45′N, 40°45′E).

[b]The name of the Regional Forestry Directorate.

Figure 1. Map showing the natural distribution (shaded areas) of *Pinus nigra* subsp. *pallasiana* in Turkey and the locations of the studied materials. The squares on the map indicate the locations of *P. nigra* subsp. *pallasiana* var. *pallasiana* stands where needles/seeds were sampled. The circle represents the seed and needle samples from *P. nigra* subsp. *pallasiana* var. *seneriana*, while the triangle indicates the seed and needle samples from *P. nigra* subsp. *pallasiana* var. *fastigiata*.

and Doyle (1990) for needle tissues. Since var. *seneriana* and var. *fastigiata* are generally sterile, seeds were available from only 7 and 8 parent trees, respectively. Initially, at least 4 megagametophytes or needle samples per parent tree in each taxon were used in DNA extraction. After DNA quantification and quality for polymerase chain reactions (PCRs) were optimized, only one DNA sample for each parent tree was used.

DNA quantification was performed with the Hoefer DyNA Quant200 Fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA), which is a filter fluorescence photometer with a fixed excitation band-pass source (365 nm) and emission band-pass filter (460 nm). The presence and the quality of the extracted DNA were also checked by running DNA in 0.8% agarose gel electrophoresis. DNA yields per megagametophyte varied from 500 to 5000 ng. All sample DNAs were diluted to 3 ng/µL for PCR applications.

2.3. Primer design and PCR conditions

The *trn*A regions used in this study are composed of the intron of *trn*L (Leu) gene, a flanking intergenic spacer, i.e. *trn*L-F, and the intron of *trn*V (Val). The *trn*L gene consists of 2 highly conserved exons, in which both flanks are also quite conservative, whereas the central part is highly variable (Bakker et al., 2000). Similarly, the *trn*V gene consists of 2 exons which are split by an intron. Three sets of primers were used to amplify the studied *trn*A region in PCR. The primer sequences for the noncoding *trn*L region of *trn*A were CGA AAT CGG TAG ACG CTA CG (forward) and GGG GAT AGA GGA CTT GA AC (reverse), while the primer sequences for *trn*L-F intergenic region of *trn*A were GGT TCA AGT CCC TCT ATC CC (forward) and ATT TGA ACT GGT GAC ACG AG (reverse) (Taberlet et al., 1991). For the *trn*V region, the primers of GTA GAG CAC CTC GTT TAC AC (forward) and CTC GAA CCG TAG ACC TTC TC (reverse) were adapted from Wang et al. (1999). The PCR reaction mixture for *trn*L and *trn*V regions contained from 5 to 50 ng/µL template DNA, 1X buffer (GeneMark Technology Co. Ltd), 1 unit of Taq DNA polymerase (GeneMark), 0.2 mM of each deoxyribonucleotide (dNTP, GeneMark), 2.5 mM $MgCl_2$, and 200 pmol of each primer in a total volume of 50 µL of PCR reaction. For the *trn*L-F intergenic spacer region, the PCR conditions were the same as above except for the primer concentration, which was 100 pmol for each primer in the PCR mixture. The PCR cycles for DNA amplifications were: initial denaturation step of 5 min at 95 °C; 35 cycles of denaturation step at 94 °C for 30 s, annealing step at 55 °C for 30 s for the primers of *trn*L and *trn*V regions and 60 °C for the *trn*L-F intergenic spacer region, and extension step at 72 °C for 50 s. The last cycle was followed by a holding step at 72 °C for 5 min. Amplification products were visualized on 1% agarose gel after staining with ethidium bromide (0.25 mg/mL) for 30 min.

2.4. Sequencing, data collection, and analysis of sequence data

The sequencing reactions for both forward and reverse primers of *trn*L and intergenic spacer *trn*L-F and *trn*V regions were carried out at the Refgen Biotechnology facilities (Middle East Technical University, Teknokent, Ankara). After data collection, 33 sequences for *P. nigra* subsp. *pallasiana*, 21 for var. *seneriana*, and 17 for var. *fastigiata* from the forward and reverse primers were aligned and checked with the DNA Baser v.3.2.0 (2012) for accuracy of the base-call. After having contig sequences and concatenating 3 *trn* regions for each sample, the *trn* sequences from Anatolian black pine and its varieties as well as other pine species from GenBank along with the outgroup were aligned with multiple sequence comparison using the log-expectation approach (MUSCLE software; Edgar, 2004). Molecular diversity parameters such as conserved sites, variable sites, parsimony-informative sites, GC content, evolutionary divergence, and genetic distance were estimated using the MEGA5 software (Tamura et al., 2011). The genetic divergence between Anatolian black pine and its varieties were computed according to the maximum composite likelihood model using 71 sequences from all seed and needle sources, where all positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. As sister groups, total *trn* sequences of 20 *Pinus* species were obtained from GenBank including 16 from section *Pinus* and 4 from section *Pinea* (Price et al., 1998; Richardson, 1998). As an outgroup, 1 *trn* sequence of *Picea orientalis* obtained from Northeast Turkey was used. Before the phylogenetic analysis, the Tamura 3-parameter model was determined as the best-fit DNA substitution model with the maximum likelihood estimation approach by using MEGA5 software (Tamura et al., 2011). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.05 for *trn*L and *trn*V; 0.68 for *trn*L-F) (Tamura, 1992).

Finally, the phylogenetic analysis was carried out with 12 *trn* sequences from Anatolian black pine and var. *seneriana* (seeds = 6 *trn* sequences, needle tissues = 6 *trn* sequences), 7 from var. *fastigiata* (seeds = 3 *trn* sequences, needle tissues = 4 *trn* sequences), and 21 from other pine species and from the outgroup. The phylogenetic tree construction was performed with the maximum likelihood (ML) approach and tested with the bootstrap method (1000 replications) using MEGA5 software (Tamura et al., 2011).

3. Results

The total length of 3 regions of *trn*A was 1163 bp with 35.05% GC content in Anatolian black pine and its 2 varieties. The lengths of *trn*L, *trn*L-F, and *trn*V were about 420 bp, 347 bp, and 396 bp. The whole region in both *trn*L and *trn*V is conserved in all studied samples from Anatolian black pine and its varieties. Only 2 variable sites (also parsimony-informative) for var. *seneriana* and 1 for var. *fastigiata* and *P. nigra* subsp. *pallasiana* revealed the *trn*L-F region (Tables 2 and 3A).

Table 2. Estimated molecular diversity parameters for *trnL*, *trn*L-F, and *trn*V gene regions for *P. nigra* subsp. *pallasiana* var. *pallasiana* (PNP), *P. nigra* subsp. *pallasiana* var. *seneriana* (PNPS), and *P. nigra* subsp. *pallasiana* var. *fastigiata* (PNPF).

Alignment site	PNP	PNPS	PNPF	All PNP including outgroup (20 *Pinus* spp. and *Picea orientalis* from GenBank)
Number of sequences	33	21	17	92
Sequence length (*trn*L + *trn*L-F + *trn*V)	1163	1163	1163	1313
Sequence length (*trn*L-F)	347	347	347	383
GC content range (%)	35.1	35.1	35.4	34.9
	28.2*	28.0*	27.8*	28.1*
Number of conserved sites	1162	1161	1162	1211
	346*	345*	346*	312*
Number of variable sites	1	2	1	102
				55*
Number of parsimony-informative sites	1	2	1	31
				15*
Nucleotide diversity	0.0015	0.0027	0.0015	0.004
				0.0091*

*: Data from *trn*L-F region.

Table 3A. *trnL-F* region: position of parsimony-informative sites in 3 *trn* regions of *P. nigra* subsp. *pallasiana*, its varieties, and outgroups. To show the variable sites, a total of 12 *trn* sequences of Anatolian black pine and its varieties were selectively used.

Section	Species	35	72	95	137	144	154	163	249	324	331	348	365	368	372	377
Pinus	PNP[a]-M[b]-S[c]52	G	-	A	C	T	C	C	G	G	G	G	A	A	A	T
	PNP-M-S75	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPS-G-S1	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPS-G-S36	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPF-T-S1	.	-	.	.	.	.	.	A	.	.	.	.	.	.	.
	PNPF-T-S21	.	-	.	.	.	.	.	A	.	.	.	.	.	.	.
	PNPF-T-N1	.	-	.	.	.	.	.	A	A	.	.	.	.	.	.
	PNPF-T-N8	.	-	.	.	.	.	.	A	A	.	.	.	.	.	.
	PNPS-G-N1	.	-	.	.	.	.	.	.	A	.	A	.	.	.	.
	PNPS-G-N14	.	-	.	.	.	.	.	.	A	.	A	.	.	.	.
	PNP-G-N1	.	-	.	.	.	.	.	.	.	.	A	.	.	.	.
	PNP-G-N16	.	-	.	.	.	.	.	.	.	.	A	.	.	.	.
Pinus	*P. nigra* JN854179 AB019891	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. densata JN854209 AB097787	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. densiflora JN854210 AB019888	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. fragilissima JN854200	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. hwangshanensis JN854194 AB019886	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. kesiya JN854191 AB019887	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. mugo JN854181 AB097793	.	-	.	.	.	A	.	.	.	.	.	G	G	G	G
	P. massoniana JN854185 AB019889	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. resinosa FJ899556 AB063600	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. sylvestris AF543753 AB097792	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. taiwanensis JN854157 DQ157880	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. thunbergii FJ899562	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
	P. tropicalis JN854156 AB063594	.	-	.	.	.	?	.	.	.	.	.	G	G	G	G
	P. uliginosa AF543755 AB097793	.	-	.	.	.	A	.	.	.	.	.	G	G	G	G
	P. yunnanensis JN854151 AB019890	.	-	.	.	.	.	.	.	.	.	.	G	G	G	G
Pinea	*P. brutia* JN854224 AB019894	.	T	.	.	C	.	A	.	.	T	.	G	G	G	G
	P. halepensis JN854197 AB019893	.	T	.	.	C	.	.	.	.	T	.	G	G	G	G
	P. heldreichii JN854195 AB019895	.	C	.	.	C	.	.	.	.	.	.	G	G	G	G
	P. pinaster FJ899583 AB019892	T	C	T	T	C	.	.	.	.	T	.	G	G	G	G
	P. pinea JN854173 AB019896	.	-	T	T	C	.	A	.	.	.	.	G	G	G	G
Outgroup	*Picea orientalis* from Turkey	T	-	C	.	.	.	T	A	T	.	.	G	.	G	-

The 3 informative sites of *P. nigra* and its varieties were located at the 249th, 324th, and 348th positions of the *trn*L-F region (Table 3A). The variable site at the 249th position of *trn*L-F was present in both megagametophyte and needle tissues of var. *fastigiata* while the variable site at the 324th position was present in the needle tissues of both var. *seneriana* and var. *fastigiata*. The variable site at the 348th position exists only in needle tissues of var. *seneriana* (Table 3A). Although the sequences of the *trn*L and V regions in sections *Pinus* and *Pinea* revealed significant variable informative sites, these 2 regions were remarkably conserved in *P. nigra* and its varieties (Table 3B).

Table 3B. *trn*L and *trn*V regions: position of parsimony-informative sites in 3 *trn* regions of *P. nigra* subsp. *pallasiana*, its varieties, and outgroups. To show the variable sites, a total of 12 *trn* sequences of Anatolian black pine and its varieties were selectively used.

Section	Species	*trn*L																								
		*trn*V																								
		50	81	96	110	111	112	116	121	123	164	267	280	285	290	304	34	57	88	134	252	295	327	393
Pinus	PNP[a]-M[b]-S[c]52	T	T	A	A	T	A	C	C	C	C	A	C	T	G	T	T	C	T	G	A	A	A	G
	PNP-M-S75	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPS-G-S1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPS-G-S36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPF-T-S1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPF-T-S21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPS-G-N1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNPS-G-N14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNP-G-N1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	PNP-G-N16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Pinus	*P. nigra* JN854179 AB019891	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. densata JN854209 AB097787	.	.	.	.	.	.	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. densiflora JN854210 AB019888	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.
	P. fragilissima JN854200	.	.	.	.	.	C	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. hwangshanensis JN854194 AB019886	.	.	.	.	.	.	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. kesiya JN854191 AB019887	.	.	.	.	.	.	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. mugo JN854181 AB097793	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. massoniana JN854185 AB019889	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. resinosa FJ899556 AB063600	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. sylvestris AF543753 AB097792	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.
	P. taiwanensis JN854157 DQ157880	.	.	.	.	.	C	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. thunbergii FJ899562	.	.	.	.	.	.	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. tropicalis JN854156 AB063594	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. uliginosa AF543755 AB097793	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P. yunnanensis JN854151 AB019890	.	.	.	.	.	.	A	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Pinea	*P. brutia* JN854224 AB019894	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	.	.	.
	P. halepensis JN854197 AB019893	G	.	.	.	G	.	A	.	.	.	.	T	.	.	.	G	.	.	.	G	.	.	.
	P. pinaster FJ899583 AB019892	G	.	T	.	G	.	.	T	.	.	.	.	.	.	.	G	.	.	T	G	.	.	T
	P. heldreichii JN854195 AB019895	G	.	T	.	G	.	.	.	.	.	.	.	.	T	.	G	.	.	.	G	.	.	T
	P. pinea JN854173 AB019896	G	.	T	.	G	.	.	T	.	.	.	.	.	.	.	G	.	.	T	G	.	.	.
Outgroup	*Picea orientalis* from Turkey	G	.	.	C	.	G	.	.	.	A	.	T	C	T	G	G	.	C	.	G	.	T	T

[a]PNP, PNPS, and PNPF stand for *Pinus nigra* subsp. *pallasiana* var. *pallasiana*, *P. nigra* subsp. *pallasiana* var. *seneriana*, and *P. nigra* subsp. *pallasiana* var. *fastigiata*, respectively.
[b]Seed source location: M = Mengen-Daren, G = Gerede-Çaydurt, and T = Tavşanlı-İkizoluk.
[c]The letter S followed by the numbers means that DNA sequence was obtained from seed megagametophytes, while the letter N followed by the numbers indicates that DNA sequence was obtained from needle tissues. Numbers represent the codes for sampled trees.

When the total length of 3 *trn*A regions of the species was compared with the other related pines, there were 2 indels (the first of 3 bp and the second of 5 bp in length) located in the 85th to 87th and 203rd to 207th positions of the *trn*L-F region. The conserved sites ranged from 1161 to 1162 among Anatolian black pine and its varieties. The parsimony-informative sites, which are defined as the nucleotide sites possessing at least 2 types of nucleotides and at least 2 of those occurring with a minimum frequency of 2, varied from 1 to 2. All base substitutions were due to transition in the parsimony-informative sites. The nucleotide diversity was higher in var. *seneriana* (0.0027)

than var. *fastigiata* and *P. nigra* subsp. *pallasiana* (0.0015) (Table 2). In fact, among the 3 studied *trn*A regions, only the *trn*L-F region had variable sites and parsimony-informative sites in Anatolian black pine and its varieties. When all 3 regions were analyzed, even with inclusion of outgroups, half of the variable and parsimony-informative sites were within the *trn*L-F region.

The phylogenetic tree constructed with the ML approach using sequences from Anatolian black pine, its varieties, species of 2 *Pinus* sections (*Pinus* and *Pinea*), and *Picea orientalis* as an outgroup revealed 3 major clusters. One of them, as expected, consisted of only the outgroup *Picea orientalis*. The other 2 groups were sections *Pinea* and *Pinus*. Within the section *Pinus*, the majority of the pine species ended up in the same subcluster while *P. nigra* subsp. *pallasiana* and its varieties were externally joined to this group, although the bootstrap values were not very strong (Figure 2). The sequences from needle tissues of var. *seneriana* and var. *fastigiata* formed especially distinct subclusters within the major *Pinus* cluster.

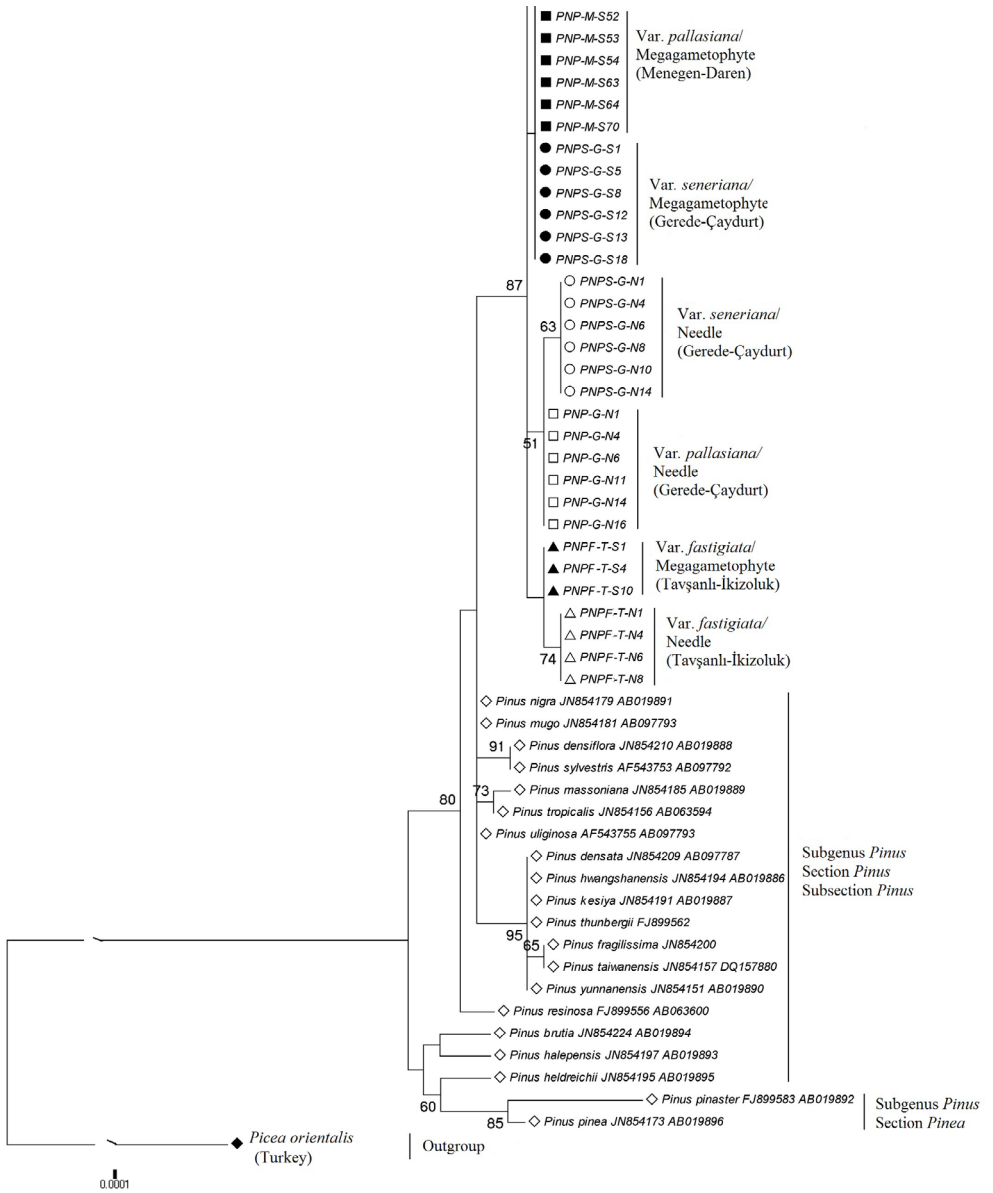

Figure 2. Phylogenetic tree constructed with *Pinus nigra* subsp. *pallasiana* var. *pallasiana* (seed megagametophyte = ■, needle = □), *P. nigra* subsp. *pallasiana* var. *seneriana* (seed megagametophyte = ●, needle = ○), *P. nigra* subsp. *pallasiana* var. *fastigiata* (seed megagametophyte =▲, needle = Δ), and sister groups [sections *Pinus* and *Pinea* (◊) and *Picea orientalis* (♦) as outgroup *trn* sequences] (the nucleotide sequences from 3 *trn* regions of cpDNA for each taxa were concatenated before analysis). Please see Tables 1 and 3 for explanations for the codes of sequences from *P. nigra* subsp. *pallasiana*. The values above branches are the bootstrap values. Only bootstrap values greater than 50% are provided.

Genetic distances between the needle tissues of var. *seneriana* and the seed megagametophytes of var. *fastigiata* as well as between the needle tissues of var. *fastigiata* and *P. nigra* subsp. *pallasiana*, estimated with the Tamura 3-parameter model (Tamura, 1992), were the highest (0.0027). Although genetic distances between the varieties and *P. nigra* subsp. *pallasiana* were high enough to be separated from each other, the genetic distances between both varieties and *P. nigra* subsp. *pallasiana* were low when the *trn* sequences were obtained from seed megagametophytes (Table 4).

4. Discussion

The lengths of *trn*L, *trn*L-F, and *trn*V in *P. nigra* subsp. *pallasiana* were within the range reported by previous studies (Wakasugi et al., 1994; Stech et al., 2003). However, the length variation in the *trn*L-F and *trn*V regions is reported frequently by previous studies (Chen, 2002; Ferri, 2009) based on whether or not the amplified and sequenced regions include exonic regions from the 5′ and 3′ ends. Chen (2002) reported that the plant *trn*L-F intergenic spacer is less than 500 bp long. When the 2 regions of 5′ and 3′ corresponding to the exons were excluded in the same study, the actual sizes of the intergenic regions in loblolly, short leaf, and slash pine were reduced to 430 bp, 427 bp, and 426 bp, respectively.

In general, *Pinus* species appear to have highly conserved *trn* regions (Aizawa et al., 2012) when they are compared with *Picea* A.Dietr. (Ran et al., 2006). Among the studied 3 regions of the chloroplast genome, the section *Pinus*, which includes *Pinus nigra* subsp. *pallasiana*, showed more parsimony-informative sites in *trn*L-F and *trn*L than in *trn*V. The other close pine section, *Pinea*, seems to be highly variable in all 3 regions. Although the section *Pinus* appeared to be highly conserved with respect to the *trn*L-F region, all the variable sites in *P. nigra* subsp. *pallasiana* were present in this region. This species, including varieties, had 1 deletion and 3 variable sites with transition of G to A. Additionally, there were 4 sites with base substitution (3 of them from G to A, 1 of them from G to T). These variable sites greatly facilitated the separation of *P. nigra* subsp. *pallasiana* varieties from the other member of the section *Pinus*. The parsimony-informative site located at the 249th position of *trn*L-F was specific to var. *fastigiata* and present in both needle and seed megagametophyte tissues. The variable sites at the 324th and 348th positions were present in *P. nigra* subsp. *pallasiana* var. *pallasiana* and in only needle tissue, which characterized the variety. Interestingly, needle tissues sampled from *P. nigra* subsp. *pallasiana* var. *pallasiana* and var. *seneriana* from the same location had the variable site at the 348th position, while seed megagametophytes from the same trees in the same location did not have this variable site. The variable site at the 324th position was also specific to the needle tissues of both var. *fastigiata* and var. *seneriana*. The constructed phylogenetic tree using the sequences from the section *Pinus* including *P. nigra* subsp. *pallasiana* and section *Pinea* revealed a clear separation of *P. nigra* subsp *pallasiana* varieties from these taxonomically related sections (Price et al., 1998; Richardson, 1998). Although the clusters had low bootstrap values, the sequences derived from both var. *seneriana* and var. *pallasiana* were grouped in separate clusters within *P. nigra* subsp. *pallasiana*. When *trn* sequences of seed megagametophytes from varieties of Anatolian black pine were used as a DNA source, there was no divergence among Anatolian black pine varieties (var. *pallasiana*, var. *seneriana*, and var. *fastigiata*). The presence of these *trn*L-F variable sites in only needle tissues of 2 varieties suggests that the divergence of these varieties from the species and the section *Pinus* may be a recent one. A clear separation of var. *seneriana* and var. *fastigiata* from var. *pallasiana*, when needle tissues were used, indicated that the variable sites in the DNA sequence of the *trn*L-F region may be

Table 4. Genetic divergence values calculated using the maximum composite likelihood method. PNP, PNPS, and PNPF stand for *Pinus nigra* subsp. *pallasiana* var. *pallasiana*, *P. nigra* subsp. *pallasiana* var. *seneriana*, and *P. nigra* subsp. *pallasiana* var. *fastigiata*, respectively. The letters following PNP, PNPS, and PNPF stand for seed source location: M = Mengen-Daren, G = Gerede-Çaydurt, and T = Tavşanlı-Ikizoluk.

Taxa	PNPS-G-S	PNPS-G-N	PNP-M-S	PNP-G-N	PNPF-T-S
P. nigra subsp. *pallasiana* var. *seneriana* /Seeds (PNPS-G-S)	-				
P. nigra subsp. *pallasiana* var. *seneriana* /Needles (PNPS-G-N)	0.0018	-			
P. nigra subsp. *pallasiana* var. *pallasiana* /Seeds (PNP-M-S)	0.0000	0.0018	-		
P. nigra subsp. *pallasiana* var. *pallasiana* /Needles (PNP-G-N)	0.0009	0.0009	0.0009	-	
P. nigra subsp. *pallasiana* var. *fastigiata* /Seeds (PNPF-T-S)	0.0009	0.0027	0.0009	0.0018	-
P. nigra subsp. *pallasiana* var. *fastigiata* /Needles (PNPF-T-N)	0.0018	0.0018	0.0018	0.0027	0.0009

present in only the true form of the variety, which could be very useful for characterization of true varietal types. The reason for detecting the variable sites in DNA sequences of *trn*L-F of needle tissues of var. *seneriana* and var. *fastigiata* but not finding them in seed megagametophytes of all studied varieties could be the paternal inheritance of the chloroplast genome in pines, since pollens are dispersed by wind over very long distances.

Considering the haploid nature of seed megagametophytes and its paternal inheritance in *Pinus* (Wagner et al., 1987; Neale and Sederoff, 1989; Wagner et al., 1989, 1992; Dong et al., 1992), the observation of nondivergence of var. *seneriana* and var. *fastigiata* from var. *pallasiana* when sequences from megagametophytes are used may be expected. This nondivergence could be explained by the fact that either these var. *seneriana* and var. *fastigiata* trees with seed production are not true varieties, or that the varietal forms are caused by a point mutation in gene(s) with pleiotropic effects involved in apical dominancy of Anatolian black pine. This point mutation may result in multiple stem with shorter needles, shorter stem with reduced internodes, and increased branch formations. Although both explanations could be justified, further studies dealing with a mutated gene involved in embryo development could be very useful to understand whether somatic mutations occur at the zygotic, embryonic, or later stages.

Despite very low frequency of both var. *seneriana* and var. *fastigiata* forms in natural Anatolian black pine stands, high frequencies of these forms are found in some areas such as Gerede-Çaydurt for var. *seneriana* and Tavşanlı-İkizoluk for var. *fastigiata*. These varietal forms are generally propagated clonally, though var. *fastigiata* produces relatively more viable seeds than var. *seneriana* (Yücel, 2000). When these varieties are propagated with seeds, however, the frequency of seedlings with varietal forms is very low. In the future, it is expected to see an increase in frequency of both varietal forms in natural forests since they are favored by forest managers during logging. However, without human assistance in propagation, it will be difficult for them to spread, since true forms of both varieties are quite sterile. Seed-producing ones must be heterozygotes, so they show segregation from varietal forms when the seedlings obtained from seeds obtain cone-producing varietal forms. However, this has to be verified with further studies dealing with control crosses and segregation analysis using DNA markers.

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