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Chloroplast DNA variation and pollen contamination in a *Pinus brutia* Ten. clonal seed orchard: implication for progeny performance in plantations

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Abstract: Pollen contamination is one of the important factors affecting the yield, adaptability, and genetic quality of the seed produced from seed orchards in forest tree breeding programs. Incoming pollen from the forests surrounding the seed orchard is a major concern in tree breeding because it contributes to losses in the expected genetic gains from seed orchard crops. The genetic variation and the level of pollen contamination in a 16-year-old *Pinus brutia* Ten. first-generation clonal seed orchard was studied using chloroplast microsatellite markers (cpSSRs). In total, 23 alleles and 36 unique allelic combinations (haplotypes) were detected based on the 6 cpSSR loci analyzed. The haplotypic diversity of the clones in the seed orchard was found to be 0.849. Out of 300 embryos analyzed, 87 were not compatible with any male parent within the seed orchard. Thus, 29% of the embryos were sired by pollen sources outside the orchard (i.e. apparent contamination). Microsatellite-based analysis revealed that the estimated contamination rate was 39.3%. Background pollination at this level will cause losses of 20% in the expected genetic gains. Our findings are valuable for the assessment of the intended seed orchard function, i.e. provision of genetically improved seed. It may be worthwhile to use pollen management strategies like strobilus stimulation, controlled pollination, and supplemental mass pollination to decrease pollen contamination and increase the genetic quality of the seeds produced.

Key words: Chloroplast microsatellite locus, genetic markers, gene flow, clonal seed orchard, Turkish red pine

1. Introduction

Turkish red pine (*Pinus brutia* Ten.) is an important forest tree species in Turkey for various economic and ecological reasons. It occurs in the eastern Mediterranean basin (mainly in the coastal strip along the Mediterranean Sea in southern Turkey), in the eastern part of the Aegean region, sparsely along the shore of the western and central parts of the Black Sea in Turkey, in the northeastern part of Greece, on the islands of the eastern Aegean Sea, and on the islands of Crete and Cyprus, as well as in Syria, Lebanon, and Iraq (Mirov, 1967; Ne'eman and Trabaud, 2000; Fady et al., 2003; Boydak, 2004; Boydak et al., 2006). *P. brutia* occupies approximately 5.9×10^6 ha of forest land in Turkey (OGM, 2012). It occurs most abundantly at altitudes up to 1300 m above sea level, where it forms pure forests or mixed forests with *Cupressus sempervirens* L., *Juniperus oxycedrus* L., *J. excelsa* M.Bieb., and a variety of angiosperm trees and shrubs. Because of its drought resistance it is widely used for afforestation of degraded areas in the Mediterranean region and similar climates. It is an important tree due to its wood being used for many industrial purposes. More specifically, its wood is chosen because of its long and suitable pulp production wood fibers, and it is extensively used for production of high-quality paper (Boydak et al., 2006). Thus, *P. brutia* has become one of the most intensively genetically improved forest tree species in Turkey. In different regions of Turkey, 68 *P. brutia* seed orchards were established from 1976 through 2010 by the Forest Tree Seeds and Tree Breeding Research Directorate.

Seed orchards are established in order to obtain genetically high-quality seeds and seedlings for reforestation and afforestation purposes (Buiteveld et al., 2001; Zobel and Talbert, 2003; Moriguchi et al., 2008). In other words, seed orchards, established with selected materials from plus trees and isolated from other populations of the same species, are expected to easily and economically provide large amounts of seed with high genetic value (Kang et al., 2001, 2004). The seed orchard is an important tool for tree breeders to change the genetic structure of forest populations in the desired direction. The production of genetically superior seeds in the seed orchard through panmictic reproduction is important. In an ideal seed orchard, panmixia is fulfilled when the following are

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present: (1) completely random fertilization (including lack of incompatibility), (2) equal number of male gametes per clone for all the seed orchard clones, (3) equal number of female gametes per clone for all the seed orchard clones, (4) no fertilization from alien pollen, (5) all seed orchard clones having equal self-fertility for all clones in the seed orchard, and (6) lack of genetic barriers affecting embryo viability (Codesido et al., 2005). If individuals in seed orchards do not reproduce panmictically, some reduction in the expected genetic gains will be observed (Harju and Muona, 1989). Fertilization by alien pollen is one possible reason for loss of the expected genetic gain. Pollen gene flow and selfing are important factors affecting yield and the genetic quality of seeds in a seed orchard (Fast et al., 1986). Therefore, the estimation of pollen contamination is of great importance for the evaluation of the effectiveness of seed orchard management strategies to reduce pollen flow from other populations, the evaluation of the seed orchard's function, and the determination of genetic gain (Torimaru et al., 2009). Previous reports have described pollen contamination using different methods in conifer seed orchards. Some have used multilocus allozyme markers to estimate pollen contamination in a number of conifer species (e.g., Paule et al., 1993; Pakkanen et al., 2000; Kaya et al., 2006). DNA-based genetic markers have also been widely used for assaying genetic variation of different forest tree species, paternity analysis, and the estimation of pollen-mediated gene flow (Buiteveld et al., 2001; Plomion et al., 2001; Goto et al., 2002; Torimaru et al., 2009). Although nuclear microsatellite (nSSR) loci have a higher number of alleles than chloroplast microsatellite (cpSSR) loci, due to their paternal inheritance in conifers cpSSRs are useful markers for the clonal identification of ramets, especially in the determination of mislabeled ramets in clonal seed orchards (Dzialuk and Burczyk, 2004) and also for estimating pollen contamination level.

The level of incoming pollen flow into the seed orchards of wind-pollinated forest trees ranges from 20% to 90%, depending on the species, age and size of the seed orchards, their pollen production, and their isolation from natural populations (Burczyk et al., 2004a; Kaya et al., 2006; Fernandes et al., 2008; Torimaru et al., 2009). To the best of our knowledge, the related research on *P. brutia* is scarce; only one study has been carried out on the estimation of pollen contamination in a *P. brutia* seed orchard in Turkey using multilocus allozyme markers, and a high (85.7%) pollen contamination rate was estimated (Kaya et al., 2006).

The main aim of this study is to estimate the genetic variation and pollen contamination rate in a first-generation clonal seed orchard of Turkish red pine (*Pinus brutia* Ten.) by using cpSSRs and to provide information that will be significant for developing seed orchard management studies and for evaluating the impact of contamination on the expected gain from the seed orchard crops.

2. Materials and methods

2.1. Seed orchard and plant materials

The first-generation clonal seed orchard under study is located near Antalya in southern Turkey (37°01′33″N, 30°32′57″E, 320 m altitude). It covers 17.8 ha and consists of 30 clones (2200 ramets in total) originating from the Gündoğmuş-Eskibağ seed stand in Antalya located at 32°08′00″N, 36°44′13″E and 1000 m altitude. The orchard was established in 1992 using 8 × 8 m spacing between trees (ramets). The whole orchard was considered as a single block and approximately 73 ramets of a given clone are randomly distributed in the orchard, provided that there should be at least 5 other ramets belonging to different clones in all directions of a given ramet. Thus, the distance between 2 ramets belonging to the same clone is at least 40 m. Such an arrangement of a seed orchard is known as a systematic arrangement (Van Buijtenen, 1971; Zobel and Talbert, 2003). There were no naturally regenerated trees in the seed orchard site. The orchard is surrounded by a natural (wild) *P. brutia* stand on the northern and northwestern sides that covers approximately 10 ha, about 170 m from the orchard's edge. However, the trees in the natural population do not show homogeneous distribution. There was at least 30 m of distance among the trees within the natural population. There are also naturally growing individual *P. brutia* trees surrounding the seed orchard at distances ranging from 6 to 110 m**.** As a result, we could select 47 samples from the natural population.

In April 2008, 1 ramet from each of the 30 clones was randomly chosen in the orchard and 15–20 cones were collected from these ramets for genotyping. An additional 4 ramets from 5 randomly selected clones (thus, 5 ramets for each of these 5 clones were selected in total) were chosen in order to test whether there was any mislabeling of ramets. In total, 50 ramets belonging to 30 clones were studied (Figure). The orchard trees were 16 years old when sampled. Cones were collected from all 4 sides of the crown as well as from the upper, middle, and lower parts of the crown. In addition, cones and needles from the terminal parts of the branches were collected from 47 randomly selected trees belonging to the nearest natural *P. brutia* population in May 2008. These trees may potentially represent putative sources of pollen contamination. The sampled trees from the natural *P. brutia* population were located at a distance of at least 30 m from each other. The needles were stored at –20 °C until DNA analysis. The cones were dried and then seeds were extracted, dewinged, cleaned by hand, labeled by mother tree or ramet, and kept at 4 °C until further use.

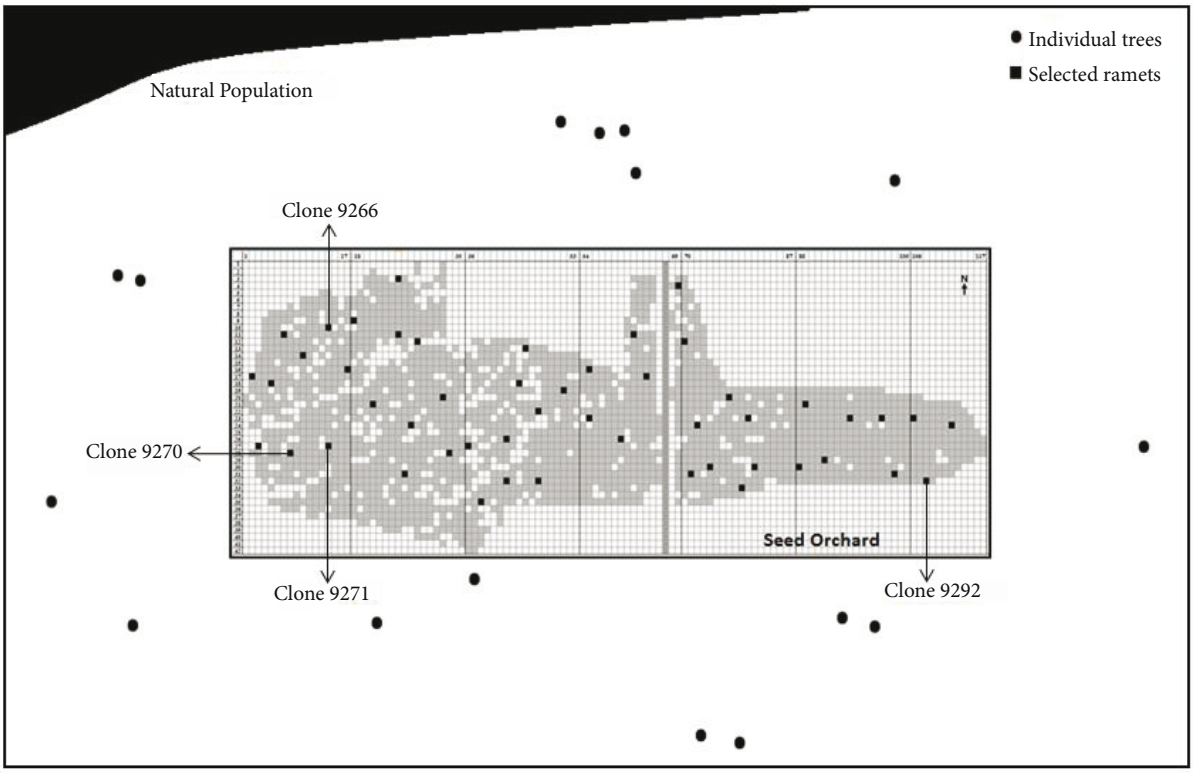

Figure. A schematic diagram showing the sampled trees from the seed orchard and relative areas of the seed orchard, natural population, and individual trees surrounding the seed orchard.

2.2. DNA isolation and cpSSR analysis

For the DNA isolation, the seeds from the orchard were germinated on moistened Whatman No. 3 filter paper in petri dishes at 24 °C for a 12-h photoperiod. The seeds were then dissected and the haploid megagametophyte and diploid embryo (about 1 cm long) tissues were put into labeled 2-mL tubes separately. DNA isolation was performed from 10 embryo tissues and a single megagametophyte for each of the clones in the orchard and from silica gel-dried needles or megagametophytes for trees of the natural population. Total genomic DNA was isolated following the Dellaporta protocol (Dellaporta et al., 1983) with slight modifications and the extracted DNA was stored at –20 °C for further use.

The genotyping of individuals was performed by 6 pairs of cpSSR primers: Pt1254, Pt15169, Pt30204, Pt41093, Pt71936, and Pt87268, originally developed for *Pinus thunbergii* Parl. (coded as by Vendramin et al., 1996). Multiplex polymerase chain reaction (PCR) was performed for 2 groups of primers (group 1: Pt1254, Pt30204, and Pt87268; group 2: Pt41093, Pt15169, and Pt71936). The DNA amplifications by PCR were carried out using the Nyx Technik Amplitronyx 6 Thermal Cycler with the following PCR profile: 5 min of denaturing at 95 °C, followed by 30 cycles of 1 min of denaturing at 94 °C, 1 min of annealing at 55 °C, and 1 min of extension at 72 °C, with a final extension of 8 min at 72 °C. The volume of the reaction mixture was 25 µL, containing 2.5 mM $MgCl_2$, 1X reaction buffer, 4 dNTPs (each 0.2 mM), 0.2 µM of each primer (forward primer FAM- or HEX fluorescent dye-labeled), and 1.5 U of Taq polymerase. The template for PCR amplification consisted of 50 ng of genomic DNA. Microsatellite fragments were scored in an ABI-PRISM 310 genetic analyzer and fragment sizes were calculated by Peak Scanner Software v.1.0 (Applied Biosystems).

2.3. Statistical analysis

For the statistical analysis of cpSSRs, length variants at each locus were combined into haplotypes due to the nonrecombining nature of the chloroplast genome. Gene diversity statistics were calculated using GENALEX version 6.3 (Peakall and Smouse, 2006) and CONTRIB 1.02 (Petit et al., 1998). The number and frequency of haplotypes in the seed orchard and background population were estimated using ARLEQUIN version 3.11 (Excoffier et al., 2005). The effective number of alleles was calculated with the formula $A_e = 1 / (1 - h) = 1 / \Sigma p_i^2$, where p_i is the frequency of the ith allele in a locus and h is the heterozygosity in a locus. Shannon's information index (I) was calculated based on

allele frequencies from the formula $I = p_i \ln p_i$ (Shannon and Weaver, 1949). The genetic diversity was estimated using the unbiased haplotypic diversity, $H_e = n\ (n-1)^{-1}\ (1 - \Sigma p_i^2)$, where p_i is the frequency of the ith haplotype (Nei, 1987).

Pollen contamination analysis requires the genotypic identification of all clones in the seed orchard. In this study, we analyzed 1 haploid megagametophyte tissue for clonal identification and embryo tissues of 10 seeds from each ramet for estimation of pollen contamination. All pollen gametes with haplotypes that could not have been produced by any of the seed orchard clones were regarded as contaminants. Such haplotypes were regarded as apparent or detected contaminants from external pollen sources. The total of detected contaminants (b) is a minimum estimate of contamination. The haplotypes of some gametes from background sources can match those produced by seed orchard clones and, thus, they will be undetected (cryptic contamination). To determine the true proportion of contamination (m), it is necessary to calculate the probability that a contaminant pollen grain has a detectable genotype (detection probability; d). If the genetic composition of the background pollen gamete gene pool is known, d can be estimated from $d = 1 - h$, where h is the frequency of indistinguishable pollen gametes, which can be produced in both the clones in the orchard and the background stand. Pollen contamination into the seed orchard from the surrounding natural populations was estimated using the multilocus estimation procedure, formulated as $m = b / d$ by Smith and Adams (1983); GENFLOW (Adams and Burczyk, 1993); and POLLEN FLOW (Slavov et al., 2005b), where b = the observed proportion of detected contaminants and d = the estimated detection probability. If the orchard seed crops result from fertilization by pollens from unimproved trees, then the predicted genetic gain is considered to be one-half of that expected under no contamination (Squillace and Long, 1981; Goto et al., 2005). The expected reduction in genetic gain under contamination (G_{RG}) is therefore $G_{RG} = [(m \times G) / 2]$, where G = the gain expected under no contamination and m = the true proportion of contamination. When G_{RG} was calculated, we assumed that G was equal to 1 and that external pollen sources originated only from completely unimproved trees.

3. Results

Considering all the studied samples of the *P. brutia* seed orchard and the background population, 1 of the 6 microsatellite loci (Pt41093) was completely monomorphic and the remaining loci were found to be polymorphic. Twenty-three alleles at the 6 cpSSR loci were identified. Table 1 shows genetic diversity estimates in the seed orchard, clones, embryos, and background population. When all the alleles were combined at the 6 cpSSR loci, 36 different haplotypes were found (Table 2). In the 30 clones of the seed orchard, 12 haplotypes were observed. Three different haplotypes [H2, H3, and H8; total frequency (f) = 0.66] were widely observed in the seed orchard (Table 2). Some clones of the orchard had the same genetic identity, i.e. the same haplotype, according to the studied 6 cpSSR loci. Most of the orchard clones' haplotypes (9 out of 12 haplotypes) were also present in the background population. Nine of the observed haplotypes were common to the seed orchard clones, embryos, and background population. Fourteen of the haplotypes observed in the embryos (39%) were not observed in either the trees of the seed orchard or of the background population. To estimate variation levels in the background pollen pool, 47 assayed trees from the surrounding stand of the seed orchard gave rise to 19 different haplotypes (Table 2). Haplotypic richness was 10.7 for the seed orchard, 16.5 for embryos, and 18 for the background population after rarefaction to the smallest sample size. Shannon's information index (I) was estimated

Table 1. Genetic diversity estimates in the *P. brutia* seed orchard clones (S.O.), embryos, and background population.

	A	A_e	N_h	R_h	I	h	H_e
S.O.	2.83 (±0.477)	1.72 (±0.272)	12	10.7	0.61 (±0.172)	0.340 (±0.102)	0.849 (±0.05)
Embryos	3.83 (±0.946)	1.90 (±0.308)	36	16.5	0.74 (±0.228)	0.378 (±0.117)	0.922 (±0.02)
Background population	3.67 (±0.843)	1.87 (±0.321)	19	18	0.72 (±0.220)	0.369 (±0.114)	0.920 (±0.04)

A = Mean number of alleles per locus, A_e = mean effective number of alleles, N_h = number of haplotypes, R_h = haplotypic richness, I = Shannon's information index, h = genetic diversity coefficient (Nei, 1987), H_e = haplotypic diversity (Nei, 1987), ±standard errors in parentheses.

Table 2. Haplotype frequencies of the *P. brutia* seed orchard clones (S.O.), embryos, and background population. Haplotype composition refers to cpSSRs Pt1254, Pt30204, Pt87268, Pt41093, Pt15169, and Pt71936, respectively.

Label	Haplotype	Frequency		
		S.O.	Embryos	Background population
H1	71 / 136 / 171 / 72 / 117 / 147	0.0600	0.1467	0.2128
H2	71 / 136 / 170 / 72 / 117 / 147	0.2800	0.1333	0.0426
H3	71 / 136 / 172 / 72 / 116 / 149	0.2000	0.1233	0.1064
H4	72 / 136 / 172 / 72 / 117 / 147	0.0800	0.0733	0.1489
H5	71 / 136 / 171 / 72 / 118 / 147	0.0200	0.0800	0.0213
H6	71 / 136 / 171 / 72 / 117 / 146	-	0.0667	0.0638
H7	70 / 136 / 171 / 72 / 118 / 147	0.0400	0.0600	0.0213
H8	71 / 136 / 172 / 72 / 117 / 148	0.1800	0.0333	0.0213
H9	67 / 136 / 171 / 72 / 117 / 143	-	0.0400	0.0213
H10	72 / 136 / 171 / 72 / 117 / 147	-	0.0300	0.0426
H11	65 / 136 / 170 / 72 / 117 / 147	-	0.0200	0.0638
H12	72 / 136 / 170 / 72 / 117 / 147	-	0.0233	0.0213
H13	71 / 137 / 172 / 72 / 117 / 147	0.0600	0.0100	0.0426
H14	71 / 136 / 170 / 72 / 117 / 146	-	0.0167	0.0426
H15	72 / 136 / 172 / 72 / 117 / 150	-	0.0200	-
H16	65 / 136 / 173 / 72 / 117 / 148	0.0200	0.0133	0.0213
H17	71 / 136 / 171 / 72 / 117 / 148	0.0200	0.0167	-
H18	70 / 136 / 171 / 72 / 117 / 148	0.0200	0.0133	-
H19	66 / 136 / 171 / 72 / 117 / 143	-	0.0100	0.0426
H20	72 / 136 / 172 / 72 / 117 / 149	0.0200	0.0067	-
H21	71 / 136 / 172 / 72 / 117 / 147	-	0.0067	-
H22	71 / 136 / 172 / 72 / 116 / 150	-	0.0033	0.0213
H23	71 / 136 / 170 / 72 / 118 / 147	-	0.0033	0.0213
H24	70 / 136 / 171 / 72 / 117 / 147	-	0.0067	-
H25	67 / 136 / 170 / 72 / 117 / 143	-	0.0033	0.0213
H26	72 / 136 / 172 / 72 / 116 / 150	-	0.0067	-
H27	70 / 136 / 172 / 72 / 117 / 148	-	0.0033	-
H28	72 / 137 / 172 / 72 / 117 / 149	-	0.0033	-
H29	69 / 136 / 170 / 72 / 117 / 148	-	0.0033	-
H30	72 / 136 / 172 / 72 / 116 / 149	-	0.0033	-
H31	66 / 136 / 171 / 72 / 117 / 147	-	0.0033	-
H32	71 / 137 / 170 / 72 / 117 / 147	-	0.0033	-
H33	72 / 136 / 173 / 72 / 117 / 150	-	0.0033	-
H34	72 / 136 / 173 / 72 / 117 / 149	-	0.0033	-
H35	71 / 136 / 173 / 72 / 116 / 148	-	0.0033	-
H36	72 / 136 / 173 / 72 / 118 / 147	-	0.0033	-

as 0.61 for the seed orchard clones and 0.72 for background population (Table 1). Unbiased haplotypic diversity (H_e) of the seed orchard clones was 0.849. Haplotypic diversity in the progeny (embryos) increased by 8.6% compared to the maternal trees of the seed orchard (H_e = 0.922) (Table 1).

Originally, it was assumed that all ramets originating from the same clone would possess identical genotypes. To test this assumption, haplotypes of 5 ramets per 5 randomly selected clones were compared with each other. Some ramets' haplotypes did not match the others that belonged to the same clone (unpublished data). Genetic identification of all orchard ramets seems to be necessary by using additional loci and by including more ramets from all clones in the future.

After genotyping 300 seeds from the 30 clones, 87 contaminant gametes were identified by their distinguishable haplotypes. Sixty-five of these contaminant gametes were from assayed trees in the surrounding stand of the seed orchard and the remaining from unknown trees. Twenty-four different haplotype identities were identified for contaminant gametes. A single matching father was found within the seed orchard for 104 gametes, and more than 1 father could be found among the 30 clones for 109 of the sampled seeds, meaning that 71% of seeds had a genetically compatible father within the orchard. However, only 3.6% of the 300 seeds were produced by a distinguishable father only present in the seed orchard. This means that 67.4% of the 300 seeds produced in the seed orchard might have been sired by the trees both in the seed orchard and in the surrounding population. Additionally, 19 mother trees had embryos pollinated by at least 30% of fathers from outside the seed orchard. Out of the 30 in the seed orchard, only 3 clones (9266, 9271, and 9292 clones), located at almost the periphery of the orchard, had the maximum number of contaminant gametes. Only one tree (clone 9270) located at the periphery of the seed orchard had not received any contaminant pollen (Figure). The minimum and maximum apparent contamination per mother tree was 10% and 50%, respectively.

The contaminant gametes were distributed across the entire seed orchard; this clearly means that the seed orchard is open to gene flow from all directions. The apparent contaminant gametes were most likely pollinated by pollen from outside the orchard, resulting in a contamination rate (b) of 0.29. The proportion of the apparent contaminants (b) is the minimum expected contamination rate. The probability that a background pollen grain carries a distinguishable multilocus marker (d) was 0.738 and microsatellite-based paternity analysis revealed that the estimated pollen contamination rate in the seed orchard (m) was 0.393. The estimated pollen contamination rate of 39.3% corresponds to a decrease in expected genetic gain from the seed orchard crops of 20%.

4. Discussion

During the past 25 years, valuable information about the gene pools of natural populations and seed orchards of different forest tree species has been provided by different researchers in different countries with the help of morphological, isozyme-based, or DNA-based analyses. Several studies about the genetic diversity, mating system, and pollen contamination of *P. brutia* populations in Turkey have been conducted using isozyme analysis (Bucci et al., 1998; Kaya et al., 2006; Kaya and Işık, 2010), RAPD analysis (Kandemir et al., 2004; İçgen et al., 2006; Lise et al., 2007, Kurt et al., 2011), ITS-2 region analysis (Tozkar et al., 2009), or cpSSR analysis (Kurt et al., 2012). In this study, we obtained valuable results for genetic structures of stands (neighboring and seed orchard) as well as valuable estimations of the level of gene flow from neighboring stands into the seed orchard using cpSSR markers.

Nei's (1987) genetic diversity coefficient (h), the observed number of alleles per locus (A), and the effective number of alleles (A_e) in the seed orchard have been found in the present study to be lower than those of the embryos and the background population. Before seed orchard establishment, the ortet trees, now existing as ramets in the orchards, were selected based on certain phenotypic traits such as height, stem diameter at breast height, and straightness. If a large fraction of the associations between molecular markers studied and these phenotypic traits were caused by linkage, then such associations could reduce the genetic diversity after the selection. Therefore, the gene pool of the seed orchard might have been narrowed, which could influence some of these genetic diversity parameters. Unbiased haplotypic diversity (H_e) was similar in the embryos (0.922) and in the background population (0.920), and both were slightly higher compared to the seed orchard (0.849). However, there are no statistically significant differences among the H_e values of the groups ($P < 0.05$). Buiteveld et al. (2001), working on a *Quercus robur* seed orchard with 57 clones and using nuclear SSR markers, estimated H_e values as 0.84, 0.72, and 0.85 in embryos and 2 background populations, respectively. Fernandes et al. (2008), working on a *Pinus pinaster* seed orchard, reported similar H_e values in the embryos (H_e = 0.784) and seed orchard clones (H_e = 0.789), but slightly lower H_e value in background population (H_e = 0.709).

In total, 36 haplotypes were identified in the present study. Several other studies on different conifer species (for example, Bucci et al., 1998, 2007; Scalfi et al., 2009) have reported that the estimates of haplotype number range between 13 and 139. The findings of the current study are in agreement with the above cited studies carried out on conifers and/or *Pinus* species. The number of haplotypes

in *P. brutia* is as high as in other pine species. Bucci et al. (1998) and Cuenca et al. (2003) found 28 haplotypes in *P. halepensis* and 27 haplotypes in *P. nelsonii*, respectively. There were fewer haplotypes (12 haplotypes) than clones (30) in the seed orchard. Such results might occur if ramets were mislabeled during establishment of seed orchard and/or if separate clones with the same haplotypic identity share common paternal ancestry (Dzialuk and Burczyk, 2004; Slavov et al., 2004). A low level of haplotype diversity of the seed orchard clones and the sharing of some of the haplotypes with the surrounding population could affect the detection probability of pollen contamination level. Therefore, it appears necessary to keep the sample size large, both in terms of orchard ramets to be genetically identified and number of loci to be studied.

Pollen contamination and inbreeding (especially selfing) are important factors that reduce the genetic efficiency of wind-pollinated seed orchards. In our study, microsatellite-based paternity analysis showed that 87 out of 300 embryos analyzed had no compatible male parent within the seed orchard, which means that the real male parents of 29% of the embryos were located outside the seed orchard. Fernandes et al. (2008) found that 108 embryos out of 206 (52.4%) had no matching genotypes within a *P. pinaster* seed orchard surrounded by *Eucalyptus grandis* and *P. pinea* as isolation trees. Buiteveld et al. (2001), Jones et al. (2008), and Torimaru et al. (2009) also found 70%, 45.9%, and 51.8%, respectively, in their studies. The presence of contaminant gametes in a seed orchard can be explained by: (1) pollen flow from outside the orchard, (2) natural regeneration in the site where the seed orchard was established, (3) scoring mistakes, and (4) some events such as heteroplasmy, inversions, and the presence of mutational hotspots (Plomion et al., 2001). Pollen flow from the neighboring natural stand of the species (approximately 170 m) together with the potential pollen flow by naturally growing individuals of *P. brutia* to the seed orchard (at a distance ranging from 6 to 110 m) could be the most likely reasons for the determination of contaminant gametes in this study. Twelve out of 87 contaminant gametes had exactly the same haplotype that was identical to the haplotype of only 1 individual tree, with a distinguishable haplotype, at a distance of 25 m from the orchard. The haplotypes of 20 other contaminant gametes corresponded to 3 trees belonging to the wild population at distances of 37 m, 103 m, and 170 m. Previous estimates of pollen contamination in seed orchards have ranged from 20% to 90% (Table 3). In this study, the estimated pollen contamination rate (m) was 0.393. The apparent alien pollen flow could originate mostly from the scattered *P. brutia* trees growing naturally within the surrounding area of the seed orchard and also from the nearby *P. brutia* stand. This level of pollen contamination falls within the range reported for several

Table 3. Estimates of pollen contamination (m) in the seed orchards of different forest tree species.

Species	Size (ha)	Marker type (no. of loci)	m (%)	Reference
Pinus taeda	2	Isozymes (7)	36	Friedman and Adams (1985)
Pinus sylvestris	12.5	Isozymes (21)	36	El-Kassaby et al. (1989)
Pinus sylvestris	16	Isozymes (21)	21	El-Kassaby et al. (1989)
Pinus sylvestris	12.5	Isozymes (21)	24–40	Yazdani and Lindgren (1991)
Pseudotsuga menziesii	2	Isozymes (11)	49	Adams et al. (1997)
Picea abies	13.2	Isozymes (11)	70	Pakkanen et al. (2000)
Quercus robur	4.5	nSSRs (6)	70	Buiteveld et al. (2001)
Pinus pinaster	11.8	cpSSRs (6)	36	Plomion et al. (2001)
Pinus thunbergii	0.5	RAPDs (28)	2	Goto et al. (2002)
Pinus brutia	11.2	Isozymes (14)	86	Kaya et al. (2006)
Pinus pinaster	4	nSSRs (3)	52	Fernandes et al. (2008)
Pinus sylvestris	13.7	nSSRs (9)	52	Torimaru et al. (2009)
Pinus koraiensis	1	nSSRs (13)	25	Feng et al. (2010)
Pinus brutia	17.8	cpSSRs (6)	39	Present study

earlier seed orchard studies in forest trees. Moreover, another study based on allozymes and carried out in another seed orchard of the same species within the same region showed 85.7% pollen contamination (Kaya et al., 2006). The pollen contamination level in the present seed orchard was much lower than that reported by Kaya et al. (2006). The high contamination level of the latter orchard reported by Kaya et al. (2006) may be due to its relatively young age (11 years old) as well as to 2 neighboring stands located about 100 m away from the studied orchard. Harju and Nikkanen (1996) estimated 48% pollen contamination in a *P. sylvestris* seed orchard isolated from other stands by about 2 km. Pakkanen et al. (2000) in *Picea abies* and Slavov et al. (2005a) in *Pseudotsuga menziesii* also estimated pollen contamination levels in 3 different years and the mean contamination level was 70% and 35.3%, respectively. Although contaminant pollen generally has a lower breeding value than pollen originating from the seed orchard clones, the assessment of genetic quality of incoming pollen from outside stands is puzzling. If the genes are introduced to a seed orchard via alien pollen that originated from populations maladapted to the habitat of the offspring establishment, gene flow may reduce the fitness of the offspring and seriously affect the survival and production of operational plantations.

Pollen contamination level depends on several factors, including the amounts of pollen production inside an orchard, the flowering synchronization among the orchard clones, the timing and duration of female cone receptivity of orchard clones relative to other pollen sources, the level of pollen production in neighboring stands, and annual weather variation (such as wind direction, temperature, and rainfall) during the period of male conelet maturation and female conelet receptivity (Harju and Muona, 1989; Burczyk et al., 2004b; Alizoti et al., 2010). The data related to annual climatic conditions prevailing over the last 10 years in the seed orchard area show that the mean monthly temperatures during the flowering season (2006) had not significantly deviated for the last 10 years, including the year of the flowering. However, the last 10 years' means of monthly precipitations were unstable during the flowering seasons of the involved years, ranging from 288.58 mm (2008) to 1410.20 mm (2012). Total annual precipitation of rain in the year of flowering (2006) was above average (1159.52 mm) (www.tutiempo.net). The timing of the flowering season for individual species varies from year to year, depending on weather conditions, and this may partly confound the relationship between pollen accumulation rates and climate conditions in individual months in the flowering year, as a given calendar month may in some years cover a larger or smaller proportion of the flowering season of a given plant species (Nielsen et al. 2010). Many reports about pollen contamination in seed orchards have demonstrated that gene flow can be extensive, and there is evidence that the pollen of widely distributed forest tree species can disperse over large distances, from 10 up to 100 km (Burczyk et al., 2004a). In this study, the per-mother-tree proportion of progenies fertilized by the pollen coming from the clones of the seed orchard ranged from 50% to 90%. This means that some factors such as flowering phenology and clonal fertility variation might cause pollination variation among mother trees. Additionally, earlier and later receptive trees in seed orchards are more prone to being fertilized by alien pollens (Harju and Nikkanen, 1996; Slavov et al., 2005a).

Although pollen contamination might increase genetic diversity in a seed orchard crop, it also seriously reduces the potential genetic gain to be obtained from the seed of the orchard (Fast et al., 1986). In addition, outside gene flow might reduce or improve the adaptability of produced seeds. The background pollination estimated in this study appears to have caused losses in the predicted genetic gains from the clonal seed orchard crop by 20%. Plomion et al. (2001) reported the pollen contamination rate as 36% in the *P. pinaster* polycross seed orchard and estimated that genetic gain was reduced by 18.25%. Kaya et al. (2006) reported that the expected genetic gain in *P. brutia* seed orchard crops was reduced by at least 43% due to a high level of pollen contamination. Flowering asymmetry and variations of pollen production among trees within the orchard were probably the main factors that cause pollen contamination and thereby reduction in the genetic gain.

In order to reduce pollen contamination and thus increase the genetic gain obtained from the seed orchard crops, some precautions should be taken. First, the establishment of seed orchards in areas well isolated from putative contamination sources can be one of the most practical methods. Second, using a greater number of ramets in wider areas might increase pollen production in the orchard. Third, selection of clones that synchronize well and produce abundant and almost equal numbers of male and female conelets might also increase pollen production in the orchard. Another approach is the reorganization of the seed orchard environment. If there are trees belonging to natural (wild) populations, as was the case in the present study, the removal of these trees could help reducing pollen contamination. Fast-growing and adaptable species to the region (i.e. *Pinus pinea*, *Eucalyptus* sp., *Cupressus* sp.) can also be planted to establish an isolation zone around the seed orchard. It may be worthwhile to apply pollen management strategies such as cone stimulation (for example, use of gibberellins to increase reproductive output), use of controlled pollination whenever possible, and supplemental mass pollination to increase the genetic quality of the seeds produced (Caron and Leblanc, 1992; Kaya et al., 2006; Stoehr et al., 2006; Fernandes et al., 2008).

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