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Genetic identification of clones and the genetic structure of seed crops in a *Pinus brutia* seed orchard

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Abstract: Megagametophyte tissue in seeds from a *Pinus bruia* Ten. seed orchard was analyzed for 9 enzyme systems (aconitase, alcohol dehydrogenase, glutamate dehydrogenase, glutamate oxaloacetate-transaminase, malate dehydrogenase, menadione reductase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase, and shikimate dehydrogenase) encoded by 14 loci using starch gel electrophoresis. Twenty-eight clones and 3 ramets per clone were sampled from the orchard for analysis. Isozyme patterns of the clones clearly indicate that each clone had a different genotype when overall loci were taken into consideration. In 5 of the 28 clones, 1 of the 3 ramets did not match the other 2 ramets at 2 or more loci. This indicates that about 6% of the studied ramets in the seed orchard were mislabeled during and/or before being planted in the orchard. The *Adh2-1* allele was unique to clone no.14; therefore, such an allele could be used to determine the extent to which a specific clone contributes to the seed crop. Of the 14 loci studied, 7 (50%) were polymorphic (0.95 criterion). Overall, 28 alleles were observed in the orchard trees and seed crops. Three of the 28 alleles (allele 2 at *Aco*, allele 2 at *Got1*, and allele 3 at *Pgi2*) were not observed in the orchard clones, but were observed only in the orchard seed crops, which indicates that these alleles originated from outside pollen sources. Important allelic differences were noted between the gene pool of the orchard clones and the gene pool of the orchard seed crops, most probably due to the presence of these alien alleles in the orchard crops. We also observed that there was 14.3%, 4.2%, and 13.5% deficiency of heterozygotes at *Mnr2, Pgd3*, and *Sdh1* loci, respectively, in the orchard seed crops. Deficiency of heterozygosity could be the result of the Wahlund effect, positive assortative mating, and/or selection for homozygotes and mating among relatives, either alone or in combination.

Key words: Multilocus, allozyme, seed orchard, *Pinus brutia*, megagametophyte

Bir *Pinus brutia* tohum bahçesindeki klonların genetik kimliği ve tohumların genetik yapısı

Özet: Bir *Pinus brutia* Ten. Tohum bahçesinden toplanan tohumların megagametofit dokusu, 14 lokus tarafından kodlanan dokuz enzim sistemi (Akonitaz, Alkol dehidrogenaz, 6-Fosfoglukonat dehidrogenaz, Fosfogluko izomeraz, Glutamat dehidrogenaz, Glutamat oksaloasetat-transaminaz, Malat dehidrogenaz, Menadion redüktaz and Şikimat dehidrogenaz) için nişasta jel elektroforezi tekniği kullanılarak analiz edildi. Analizler için tohum bahçesindeki 28 klonun her birinden üçer adet rametten tohum toplandı. Tüm lokuslar göz önüne alındığında, klonların izoenzim bantlaşma yapılarına bakınca, her bir klonun farklı genotiplere sahip olduğu görüldü. Tohum bahçesindeki 28 klondan beşinde, üç rametten birinin en azından iki ya da daha çok lokusta diğer iki ramet ile uyuşmadığı görüldü. Bu durum, tohum bahçesinde çalıştığımız 84 rametin (bireyin) yaklaşık %6'sının tohum bahçesi kurulurken ya da kurulmadan önce yanlış etiketlendiğini göstermektedir. *Adh2-1* alleli 14. klona özgü bir alleldir ve bu allel, bu klonun tohum bahçesinde üretilen

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tohumlara katkısının ne olduğunu belirlemek için kullanılabilir. Çalışılan 14 lokustan yedisi polimorfiktir. Tohum bahçesindeki klonlarda ve tohum bahçesindeki klonlar tarafından üretilen tohumlarda toplam 28 allel gözlendi. Yirmi sekiz allelden üçü (*Aco-2*, *Got1-2* ve *Pgi2-3*) bizzat klonlarda değil, sadece klonlar tarafından üretilen tohumlarda gözlendi. Bu durum, bu allellerin tohum bahçesi dışından kaynaklandığını göstermektedir. Tohum bahçesi tohumlarında bulunan bu üç allelin tohum bahçesi klonlarında bulunmaması, klonların gen havuzu ile onlardan üreyen tohumların gen havuzu arasında önemli allellik farklılıklar oluşturdu. Ayrıca, tohumlarda *Mnr2*, *Pgd3*, ve *Sdh1* lokuslarında sırasıyla %14.3, %4.2 ve %13.5 oranında heterozigotluk azlığı bulundu. Heterozigotluk azlığının nedenleri; Wahlund etkisi, benzer özelliklere sahip bireylerin kendi aralarında eşleşmesi, homozigotlar lehine seçilim ve akraba bireyler arasındaki eşleşmeden kaynaklanabilir.

Anahtar sözcükler: Çoklu-lokuslar, allozim, tohum bahçesi, *Pinus brutia*, megagametofit

Introduction

The primary purpose of tree improvement programs is to produce genetically improved seeds. Seed orchards are one of the means used to obtain maximum genetic gains in the shortest time period. The major objective of seed orchards is to produce mass quantities of genetically improved seeds. The procedure by which this can be accomplished is to bring together phenotypically superior clones (or progeny from those clones) and establish them according to a specified design, so that the chance for cross-pollination among these selected trees will be high (Weir 1981). It is also possible to investigate mating systems and pollen contamination, if any, in seed orchards.

There are 2 basic types of seed orchards: seedling seed orchards, and vegetative or clonal seed orchards (Weir 1981). Clonal seed orchards are the most common type in use and are established with a finite number of clones (ortets) replicated with a certain number of grafts (ramets) (Zobel and Talbert 1984; Ladislav 1991).

Clonal identification is a prerequisite, both for tree breeding programs and for mating system and pollen contamination studies. Mislabeling ramets or skipping some clones during establishment of seed orchards might influence the accuracy of the parameters estimated in contamination and mating systems. In particular, in the case of skipping some clones that are otherwise present in a seed orchard, the pollen gametes that belong to the skipped clones are considered to be contaminating pollen.

Pollen contamination is a major problem in seed orchards and can change the genetic composition of seed crops, reducing the genetic quality of seeds due to a contaminated pollen pool. Determination of the genetic structure of seed crops in an orchard is also important because seeds from an orchard are used for afforestation purposes.

Electrophoretic analysis of isozymes has made it possible to directly quantify genetic variation in conifers. Conifers are suitable for allozyme analysis because there are 2 types of tissue [megagametophyte (1*N*) and embryo (2*N*)] present in their seeds. If we determine the genetic make-up of both of these tissues, the pollen contribution to the embryo can be inferred. Thus, by sampling many progeny of the same clone the genetic make-up of the clones and pollen pool of that clone can be determined. Similarly, by analyzing embryo tissue in seeds from a given clone, the genetic makeup of the seed crops produced by that clone can be determined.

Molecular markers are often employed in the identification of domesticated germplasms, such as clones, varieties, or cultivars. Allozyme analysis is a suitable technique for genetically identifying a given individual of forest tree (Ladislav 1991; USDA Forest Service 2000, 2001a, 2001b). The National Forest Genetics Laboratory (NFGEL) of the USDA Forest Service in California, USA, has often used isozyme analysis for clonal identification in some species. For example, in one study 19 *Pseudotsuga menziesii* trees were genetically identified (USDA Forest Service 2002). In another study 3 individual Douglas fir specimens were also genotyped at isozyme and SSR loci to assess their clonal identity (USDA Forest Service 2003). Allozymes are also widely used to determine the genetic structure and genetic variation of populations of forest trees (Conkle 1980; Cheliak et al. 1987; Conkle et al. 1988; Adams 1993; Ladislav et al. 1993).

The objectives of the present study were as follows: 1) to determine the genetic identity of all clones in a *Pinus brutia* seed orchard and 2) to obtain information about the genetic structure of the seed crops derived from the orchard.

Materials and methods

Seed orchard and seed material

The material for this study was obtained from a *P. brutia* Ten. clonal seed orchard located in Asar, near Antalya in southern Turkey (240 m a.s.l., lat 37°01′30″N, long 30°43′30″E). The seed orchard is located about 19 km from the city center. The orchard consists of 28 selected clones originating from a seed stand located 800 m a.s.l. in Çameli-Göldağı, near Denizli in southwestern Turkey. Each clone in the orchard was represented by about 50 ramets (range: 30-90). The grafts were planted with 8 × 8-m spacing in the seed orchard in 1986 (OATIAM 1997). Three ramets per clone (thus, 28 × 3 = 84 trees) in the seed orchard were sampled for this investigation. Cones were collected in 1997 from 3 grafts (ramets) per clone, each distributed randomly in the seed orchard. The cones were first dried at room temperature, and then the seeds were extracted, cleaned, labeled, and stored according to tree. The seeds were stored at 4 °C until analysis.

Electrophoretic study

Before analysis the seeds were germinated on moistened Whatman No. 3 filter paper in petri dishes at 24 °C. The seeds were then dissected, and haploid megagametophytes (endosperm) were homogenized separately with 75 µL of 0.2 M phosphate buffer (pH 7.5) (Kara et al. 1997). The resulting homogenates were analyzed simultaneously in 4 gel buffer systems, according to slightly modified methods of Conkle et al. (1982) (Kara et al. 1997; Kaya 2001). Analyses were performed on 9 enzyme systems encoded by 14 loci using starch gel electrophoresis. Electrophoretic separation was carried out on 12.0% starch gels. The analyzed enzymes are listed in Table 1, along with their enzyme commission numbers, abbreviations, buffers used, and number of loci scored. The inheritance details of these enzymes were reported by Kara et al. (1997).

Statistical analysis

We analyzed haploid megagametophytes and embryo tissues of 16 seeds from each ramet. Thus, multilocus allozyme genotypes of all 84 sampled trees were inferred from samples of 16 megagametophytes per tree. With a sample of 16 megagametophytes per tree, the probability (P) of misclassifying a heterozygous maternal genotype at any single locus was less than 3.5×10^{-5} [i.e. $P = (1/2)^{n-1} = (1/2)^{15} = 3.5$

Table 1. The analyzed enzymes, their abbreviations, enzyme commission numbers, gel buffers used, and numbers of loci scored in *Pinus brutia.*

Enzyme name	Abbreviation	E.C. No.	Buffer*	Numbers of loci scored
Aconitase	ACO	4.2.1.3	$MC_{6.1}$	1
Alcohol dehydrogenase	ADH	1.1.1.1	TBE	1
Glutamate dehydrogenase	GDH	1.4.1.2	TC	1
Glutamate oxaloacetate-transaminase	GOT	2.6.1.1	TC	3
Malate dehydrogenase	MDH	1.1.1.37	$MC_{8.1}$	1
Menadion reductase	MNR	1.6.99.2	TBE	2
6- Phosphogluconate dehydrogenase	6PGD	1.1.1.44	$MC_{6.1}$	2
Phosphogluco isomerase	PGI	5.3.1.9	$MC_{8.1}$	2
Shikimate dehydrogenase	SDH	1.1.1.25	$MC_{6.1}$	1

* $MC_{6.1}$ = Morpholine Citrate (pH 6.1); $MC_{6.1}$ = Morpholine Citrate (pH 8.1); TBE = Tris-Borate-EDTA; TC = Tris-Citrate. The details for gel buffers used are reported by Kara et al. 1997).

× 10^{-5}]. Embryo tissue banding patterns of approximately 1344 seeds (16 seeds × 3 ramets × 28 clones) were used to determine the genetic structure of the seed orchard crop. Allozyme frequency data were used to calculate the parameters related to the determination of genetic diversity and structure (i.e. mean sample size per locus, mean number of alleles per locus, percentage of polymorphic loci, mean heterozygosity observed and expected from the Hardy-Weinberg proportion, and Wright's F-statistic). All statistical analyses were performed with the BIOSYS-1 computer program (Swofford and Selander 1981).

Results

Genetic identity of the clones

Genotypes of 84 *P. brutia* individuals (28 clones × 3 ramets) in a clonal seed orchard were identified using starch gel electrophoresis. Clonal genotypes were inferred from segregation of megagametophytes by analyzing 16 seeds of each ramet. The results show that the genotypes of 28 clones differed from each other when all loci were taken into consideration.

Originally, it was assumed that all 3 ramets originating from the same clone would possess identical genotypes. To test this assumption, genotypes of 3 ramets per clone were compared with each other for overall loci. In 5 out of the 28 clones, 1 of the 3 ramets did not match the other 2 at 2 or more loci. Table 2 shows these 5 clones with mismatching ramets.

Ramet 3 of clone 4 (4-3) did not match with 4-1 and 4-2 at *Mdh1, Pgd2*, and *Pgd3* loci. Similarly, ramet 3 of clone 6 (6-3) did not match with 6-1 and 6-2 at *Got2* and *Pgi2* loci. Ramet 2 of clone 15 (15-2) did not match with 15-1 and 15-3 at *Adh2, Mdh1, Mnr2, Pgd2, Pgd3*, and *Sdh1* loci. Ramet 2 of clone 18 (18-2) did not match with 18-1 and 18-3 at *Adh2, Got2, Mdh1, Pgd2,* and *Sdh1* loci. Finally, ramet 2 of clone 20 (20-2) did not match with 20-1 and 20-3 at *Mdh1, Pgd2, Pgi2*, and *Sdh1* loci.

Table 2. Clones (4, 6, 15, 18 and 20) that exhibit a mismatching ramet with the other 2 ramets of the same clone in the *P. brutia* seed orchard with 28 clones.

Clone No-Ramet No	*Aco*	*Adh2*	*Gdh*	*Got1*	*Got2*	*Got3*	*Mdh1*	*Mnr1*	*Mnr2*	*Pgd2*	*Pgd3*	*Pgi1*	*Pgi2*	*Sdh1*
4-1	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/2	1/2
4-2	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/1	1/1	1/1	1/2	1/2
4-3*	1/1	2/2	1/1	1/1	1/2	1/1	**1/2**	1/1	1/1	**2/2**	**1/3**	1/1	1/2	1/2
6-1	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/1	1/2	1/1	1/2	2/2
6-2	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/1	1/2	1/1	1/2	2/2
6-3*	1/1	2/2	1/1	1/1	**1/1**	1/1	2/2	1/1	1/1	1/1	1/2	1/1	**1/1**	2/2
15-1	1/1	2/2	1/1	1/1	1/2	1/1	1/1	1/1	1/2	1/2	1/1	1/1	1/2	2/2
15-2*	1/1	**1/2**	1/1	1/1	1/2	1/1	**2/2**	1/1	**1/1**	**1/1**	**2/3**	1/1	1/2	**2/3**
15-3	1/1	2/2	1/1	1/1	1/2	1/1	1/1	1/1	1/2	1/2	1/1	1/1	1/2	2/2
18-1	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/2	1/1	1/1	1/1	2/3
18-2*	1/1	**1/2**	1/1	1/1	**2/2**	1/1	**1/2**	1/1	1/1	**2/2**	1/1	1/1	1/1	**1/1**
18-3	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/2	1/1	1/1	1/1	2/3
20-1	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/3	1/3	1/1	1/1	2/2
20-2*	1/1	2/2	1/1	1/1	1/2	1/1	**1/2**	1/1	1/1	**2/2**	1/3	**1/2**	1/1	**1/2**
20-3	1/1	2/2	1/1	1/1	1/2	1/1	2/2	1/1	1/1	1/3	1/3	1/1	1/1	2/2

* Mislabeled ramets (each mismatching loci are shown in bold numbers).

Genetic structure of the seed crops in the seed orchard

Allele frequencies for the studied loci in both the clones and seed crops derived from the seed orchard are presented in Table 3. Of the 14 loci studied, 7 (50%) were polymorphic (at 0.95 criterion). Overall, 28 alleles were observed in the orchard trees and seed crops. Three of the 28 alleles (allele 2 at *Aco*, allele 2 at *Got1*, and allele 3 at *Pgi2*) were only observed in the orchard seed crops. Clearly, these alleles came from pollen gametes that were not produced by any individuals in the orchard; most probably they arose from neighboring stands. Genetic variability parameters at 14 loci in the embryos are given in Table 4. The observed mean heterozygosity of the orchard crops was 0.225, while the expected mean heterozygosity was 0.233. Although the mean number of alleles in the orchard crops was higher than that in the orchard seed trees, the observed heterozygosity in the crops was lower than in the orchard trees. There was significant deviation from Hardy-Weinberg equilibrium in the seed crop gene pool, with respect to *Mnr2, Pgd2, Pgd3*, and *Sdh1* loci ($P < 0.01$, $P < 0.022$, $P < 0.028$, and $P < 0.01$, respectively).

Table 3. Alleles frequencies at 14 loci in the orchard and the orchard crop.

Locus	Allele	Orchard	Orchard seed crop
Aco	1	1.000	0.993
	2	0.000	0.007*
Adh2	1	0.025	0.047
	2	0.975	0.953
Gdh	1	1.000	1.000
Got1	1	1.000	0.995
	2	0.000	0.005*
Got2	1	0.618	0.729
	2	0.382	0.271
Got3	1	1.000	1.000
Mdh1	1	0.318	0.307
	2	0.682	0.693
Mnr1	1	1.000	1.000
Mnr2	1	0.870	0.845
	2	0.130	0.155
6pgd2	1	0.613	0.512
	2	0.353	0.479
	3	0.034	0.008
6pgd3	1	0.627	0.645
	2	0.152	0.112
	3	0.221	0.243
Pgi1	1	1.000	1.000
Pgi2	1	0.654	0.660
	2	0.346	0.339
	3	0.000	0.001*
Sdh1	1	0.305	0.416
	2	0.553	0.462
	3	0.142	0.122

* Alleles were observed only at the orchard seed crops.

Fixation indices for the orchard crops varied from 0.024 (*Mdh1*) to 0.143 (*Mnr2*), while only 3 differed significantly from zero (*Mnr2, Pgd3*, and *Sdh1*), thereby proving 14.3%, 4.2%, and 13.5% deficiency of the heterozygotes, respectively (Table 5).

Discussion

Seed orchards are established to produce mass quantities of genetically improved seed from phenotypically superior trees. Furthermore, seed orchards provide material for progeny tests and other tree improvement studies. It is assumed that all ramets originating from a particular clone in a clonal seed orchard will possess identical genotypes; however, this assumption is not always true in clonal seed orchards, mainly due to mislabeling of ramets or skipping of some clones during the establishment of seed orchards. Such errors in a seed orchard lead to incorrect estimates of pollen contamination and can result in incorrect/undesired results for progeny tests and other tree improvement studies. In the present study we determined that 5 of the 84 ramets were mislabeled, as each ramet did not match with the other 2 tested members of the same clone at 2 or more loci.

Apparently ramet 3 of clone 4, ramet 3 of clone 6, ramet 2 of clone 15, ramet 2 of clone 18, and ramet 2 of clone 20 were mislabeled during or before the

Table 4. Genetic variability at 14 loci in the orchard and the orchard crop.

Population	Mean sample size per locus	Mean number of alleles per locus	Percentage of loci polymorphic*	Mean Heterozygosity**	
				Direct-count	Expected
Orchard trees	1653.0 (± 0.0)	1.8 (± 0.2)	50.0	0.232 (± 0.067)	0.232 (± 0.066)
Orchard seed crop	1379.1 (± 9.0)	2.1 (± 0.2)	50.0	0.225 (± 0.061)	0.233 (± 0.063)

* A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.
** Unbiased estimate (see Nei 1978).

Table 5. Wright's Fixation Indices (F) of the orchard crops.

Locus	Heterozygotes		Fixation Index (F)	χ^2	P
	Observed	Expected			
Aco	19	18.878	- 0.007	0.06	0.8086
Adh2	125	124.876	- 0.001	0.00	0.9705
Got1	15	14.925	- 0.005	0.04	0.8511
Got2	570	551.639	- 0.034	1.55	0.2133
Got3	1	1.000	0.000	0.00	1.0000
Mdh1	612	597.784	- 0.024	0.80	0.3726
Mnr2	**305*** **	**355.873**	**0.143**	**27.77**	***0.0000***
Pgd2	706	711.841	0.008	0.10	0.7529
Pgd3	**687***	**717.710**	**0.042**	**4.28**	***0.0385***
Pgi2	598	597.465	- 0.001	0.00	0.9739
Sdh1	**682*** **	**788.341**	**0.135**	**39.49**	***0.0000***

establishment of the seed orchard. The ramets that were identified as mislabeled were compared with other clones in the seed orchard. These comparisons suggest that genotypes of ramets mislabeled as 4-3, 6-3, 15-2, and 20-2 match the genotypes of clones 24, 7, 14, and 24, respectively. On the other hand, 1 genotype (the ramet mislabeled as 18-2) did not match any other clone in the seed orchard. As a result, 6% of the 84 individuals studied in the seed orchard were mislabeled during or before planting in the orchard. Unfortunately, this type of experimental error is often observed in the establishment stage of seed orchards. For example, Ladislav (1991) reported that 10 of 86 ramets (11.6%) did not completely match with the genotypes of their supposedly related clones. Mislabeled ramets should not be included in studies of seed orchards (i.e. mating system, pollen contamination, and determination of genetic structure). Consequently, we did not include these mislabeled ramets in the estimation of genetic parameters or determination of the genetic structure of the seed crops in the orchard (Kaya et al. 2006).

Seeds obtained from the seed orchard are used for afforestration by the Turkish Ministry of Forestry. Accurate knowledge of the genetic structure of seed crops from seed orchards is important for obtaining greater genetic improvement in these forests. In particular, it is desirable that seeds obtained from seed orchards reflect the genetic structure of the clones in the orchards. Pollen contamination from outside sources decreases the genetic quality of seed orchard crops, especially if outside pollen sources are genetically inferior to those of the seed orchard.

Important allelic differences were observed in the present study, most probably due to the presence of alleles in the orchard seed crops that were not observed in the orchard clones. Based on our allele frequency studies of loci in both the clones and seed crops in the seed orchard, 3 of the 28 alleles (allele 2 at *Aco*, allele 2 at *Got1*, and allele 3 at *Pgi2*) were observed only in the orchard seed crops (Table 3). Clearly, these alleles were not produced by any individuals in the orchard, but rather came from the pollen of neighboring stands. The *Adh2-1* allele was unique to clone no.14 and this allele could be used to determine to what extent a specific clone contributed to the seed crop. Our data show that there was significant deviation from Hardy-Weinberg equilibrium in the seed crops, with respect to *Mnr2, Pgd2, Pgd3*, and *Sdh1* loci ($P < 0.01$, $P < 0.022$, $P < 0.028$, and $P < 0.01$ respectively). Moreover, fixation indices differed significantly from zero at *Mnr2, Pgd3*, and *Sdh1*, thereby proving the deficiency of heterozygotes (14.3%, 4.2%, and 13.5%, respectively). Deficiency of heterozygosity might be the result of the Wahlund effect, positive assortative mating, selection for homozygotes, or mating among relatives (El-Kassaby et al. 1987).

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