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A Fast Method for Checking the Genetic Identity of Ramets in a Clonal Seed Orchard by RAPD Analysis with a Bulking Procedure

By S. GOTO[1]), F. MIYAHARA[2]) and Y. IDE[3])

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Summary

In this study we demonstrate a fast method for checking the genetic identity of ramets in a Japanese black pine (*Pinus thunbergii* PARL.) clonal seed orchard, using random amplified polymorphic DNA (RAPD) analysis with a bulking procedure. We used six different artificial mixtures consisting of needle samples from two clones that were bulked in the proportion three to one to test the sensitivity of RAPD markers. We compared the RAPD patterns of the bulked samples with those of the single clones used for the artificial mixtures. Out of 20 markers, 18 markers were present in the bulked samples, when one of the clones possessed the marker. However, two markers were absent in the bulked samples, even though one of the clones possessed the marker. Using the 18 markers, RAPD patterns of the bulked samples were different from those of single clones. Out of 18 markers selected in this study, we used 15 markers for checking the genetic identity of ramets in the seed orchard. First, we collected the needles of 157 trees from the seed orchard, individually. Second, we mixed an equal amount of needle samples from a maximum of four individuals of the same clone, depending on the planting map. Third, we compared the RAPD patterns of the bulked samples with those of their standard individuals of the clone. Out of 42 bulked samples (14 clones x 3 bulked samples) investigated, we found the RAPD patterns of 3 bulked samples to be different from those of the standard individual of the diagnostic clone. Subsequently, we fingerprinted a total of 12 trees comprised of 3 suspicious bulked samples with RAPD markers individually, and detected one mislabeled tree per bulked sample. We were able to check the genetic identity of 157 trees by making a RAPD analysis of 42 bulked samples and 12 individuals. The workload was only about one-third of the workload when making the individual RAPD analyses. We concluded that RAPD analysis with a bulking procedure would be useful for rapidly checking the genetic identity of ramets in clonal seed orchards.

Key words: Bulked samples, genetic identity, mislabeling, *Pinus thunbergii*, RAPD, seed orchard.

Introduction

Tree improvement strategies include the control of natural seed sources and the establishment of orchards of selected genotypes (ZOBEL and TALBERT, 1984). When properly performed, the vegetative propagation method is a powerful means of making the clonal materials consisting of a seed orchard and capturing the genetic superiority of selected individuals. However, mis-plantings and mis-labelings are unfortunately common during the establishment of seed orchards (ADAMS, 1983; HARJU and MUONA, 1989; WHEELER and JECH, 1992). Additionally, the grafted materials are often used for ramets in the seed orchard (HONG, 1975), and sometimes the rootstocks overtake the graft. As it is difficult to detect the genetic identity of ramets in clonal seed orchards by visual inspections, tools are needed for this purpose. Molecular markers have proven to be very useful in distinguishing among related genotypes. Recently developed random amplified polymorphic DNA (RAPD) markers (WELSH and MCCLLELAND, 1990; WILLIAMS *et al.*, 1990) are polymorphic within-species levels (e.g. CARLSON *et al.*, 1991; KEIL and GRIFFIN, 1994; SCHEEPERS *et al.*, 1997), and have been successfully used for distinguishing among orchard clones (VAN DE VEN and MCNICOL, 1995; KAWAUCHI and GOTO, 1999).

Despite the fact that the RAPD procedure is relatively simple and fast, its practical application is still limited in cases where large numbers of individuals need to be examined. The number of individuals in seed orchards must be large enough to allow for the desired spacing, maximum seed production, adequate pollination, and minimum of relatedness among individuals (ZOBEL and TALBERT, 1984), so a fast method for checking the genetic identity of ramets in clonal seed orchards is needed. One approach is to use a bulking procedure. DNA extractions and polymerase chain reaction (PCR) amplifications for several plants can occur in a single step with bulked

[1]) Corresponding author: SUSUMU GOTO, University Forest in Hokkaido, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yamabe, Furano, Hokkaido 079-1561, Japan.
Ph. +81-167-42-2111; Fax. +81-167-42-2689.
E-mail: gotos@uf.a.u-tokyo.ac.jp

[2]) Fukuoka Prefecture Forest Research and Extension Center, Toyoda 1438-2, Yamamoto-machi, Kurume, Fukuoka 839-0827, Japan.
Ph. +81-942-45-7983; Fax. +81-942-45-7901.

[3]) Laboratory of Forest Ecosystem Studies, Department of Ecosystem Studies, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.
Ph. +81-3-5841-5490; Fax. +81-3-5841-5494

samples. Bulking procedures have been effectively used to identify RAPD markers linked to disease-resistant genes in lettuce (MICHELMORE *et al.*, 1991) and to estimate the relatedness between alfalfa populations (YU and PAULS, 1993).

The Japanese black pine (*Pinus thunbergii* PARL.) is a very common and important forestry species, the members of which serve as wind break against sand and salt in coastal areas in Japan. However, during the last five decades the species has been severely damaged by pine-wood nematodes (*Bursaphelenchus xylophilus* [STEINER et BUHRER] NICKLE), especially in southwestern Japan. Based on the severity of the pest and the importance of the species, a research project on the selection and production of pine-wood nematode-resistant plant material was initiated in 1978. As part of this project, sixteen resistant trees were selected from 14,620 candidate trees of Japanese black pine (FUJIMOTO *et al.*, 1989), and 21 clonal seed orchards of these resistant clones were established through grafting by 1999. These seed orchards have played an important role in the reforestation of the coastal area, which was severely damaged by pine wilt disease (TODA *et al.*, 1993).

In this present study, we demonstrate a rapid method for checking the genetic identity of ramets in a Japanese black pine clonal seed orchard consisting of the nematode-resistant clones, using RAPD analysis with a bulking procedure.

Materials and Methods

Seed orchard

The seed orchard used for this study was established in 1987 in Ogoori, Fukuoka pref., Japan. The total area of the seed orchard is 0.5 ha. The ramets were multiplied by grafting and planted systematically (GIERTYCH, 1965). The spacing between trees is 5 x 5 m. There were 200 trees in the seed orchard when it was established. Each tree has an assigned number (No. 1–200) based on the initial arrangement of the orchard.

DNA extraction and PCR amplification

For each sample, DNA was extracted from needles using a modified CTAB method (SHIRAISHI and WATANABE, 1995). Extracted DNA was purified with ELU-QUIK™ DNA purification kit (Schleicher & Schuell Co.). Purified DNA was then used as template DNA for RAPD analysis. RAPD reactions were performed in 20-µl volume containing 10 ng of template DNA, 2.0 µl of 10x Stoffel buffer (100 mM Tris-HCl, 100 mM KCl, pH 8.3), 0.20 mM of each dNTP, 0.25 µM of RAPD primer, 3.0 mM of $MgCl_2$, and 1 unit of *AmpliTaq* DNA polymerase Stoffel Fragment (Perkin-Elmer Cetus). For DNA amplification, the reactions were performed in a TaKaRa PCR Thermal Cycler MP (TP-3000) using a PCR profile consisting of one cycle of 1 min. at 94°C, 45 cycles of 10 sec. at 94°C, 30 sec. at 36°C and 60 sec. at 72°C, and one cycle of 7 min. at 72°C. After amplification, the products were separated on 2.0% agarose-gel by electrophoresis and stained with ethidium bromide. The gels were then photographed on a UV-trans-illuminator.

Examination of sensitivity by bulking procedure

To check the sensitivity of RAPD markers in the bulking procedure, in this part of the study we used five clones, named as Shima-(t)64, Yoshida-(t)2, Namikata-(t)73, Obama-(t)30 and Tosashimizu-(t)63, out of 16 orchard clones. We made six different artificial mixtures (Bulk 1-6) of needles from two clones, mixed in the proportion of three to one (*Table 1*). We used a total of 20 RAPD markers, which were reproducible and polymorphic among the 16 clones. These markers were selected by GOTO (1998) and GOTO *et al.* (unpublished). We compared the RAPD patterns of the bulked samples with those of single clones used for the artificial mixtures. We checked to see if the markers possessed by fewer clones were present in the bulked sample. Only markers that were sufficiently detected in the bulked samples were used for checking the genetic identity of ramets in the seed orchard.

Table 1. – Contents of artificial mixtures used for checking the sensitivity of RAPD markers.

No.	Contents			
Bulk 1	Shima-(t)64	150mg	+ Namikata-(t)73	50mg
Bulk 2	Shima-(t)64	150mg	+ Obama-(t)30	50mg
Bulk 3	Shima-(t)64	150mg	+ Tosashimizu-(t)63	50mg
Bulk 4	Yoshida-(t)2	150mg	+ Namikata-(t)73	50mg
Bulk 5	Yoshida-(t)2	150mg	+ Obama-(t)30	50mg
Bulk 6	Yoshida-(t)2	150mg	+ Tosashimizu-(t)63	50mg

Checking the genetic identity of ramets in the seed orchard

We collected needles from 157 trees in the seed orchard in March 2000. These trees were identified as being from one of 14 clones depending on the planting map. Out of 16 orchard clones, we have already confirmed the accuracy of the individual arrangement of the rest two clones, namely Ooseto-(t)12 and Obama-(t)24, by individual RAPD analyses (data not shown). The standard individuals of the 14 clones were the same as those identified by GOTO (1998). We put the needle samples from each tree into a polyvinyl bag and noted the clone name and the assigned number depending on the planting map. Subsequently, we mixed an equal amount (50 mg) of needles from a maximum of four individuals of the same clone and made a total of 42 bulked samples (14 clones x 3 bulked samples) (*Table 2*). If RAPD patterns of the bulked sample were inconsistent with those of the standard individual of the diagnostic clone, the existence of mislabeled trees within the bulked samples was suggested. In this case, trees consisting of the suspicious bulked samples were fingerprinted with RAPD markers selected by GOTO (1998) individually.

Table 2. – Description of the bulked samples used for checking the genetic identity of ramets in the seed orchard.

Clone[a]	Bulked samples[b]	Total[c]
Namikata-(t)73	A (4), B (4), C (2)	10
Obama-(t)30	A (4), B (4), C (4)	12
Tosashimizu-(t)63	A (4), B (4), C (3)	11
Ooita-(t)8	A (4), B (4), C (4)	12
Ei-(t)425	A (4), B (4), C (3)	11
Tsuyazaki-(t)50	A (4), B (4), C (4)	12
Misaki-(t)90	A (4), B (4), C (3)	11
Namikata-(t)37	A (4), B (4), C (3)	11
Tanabe-(t)54	A (4), B (4), C (4)	12
Yasu-(t)37	A (4), B (4), C (2)	10
Shima-(t)64	A (4), B (4), C (4)	12
Yoshida-(t)2	A (4), B (4), C (3)	11
Sendai-(t)290	A (4), B (4), C (4)	12
Mitoyo-(t)103	A (4), B (4), C (2)	10
Total		157

[a]) Clone name depending on the planting map.

[b]) The name of the bulked sample and the number of individuals consisting of each bulked sample in parenthesis.

[c]) A total number of individuals used for checking the genetic identity for each clone.

Results

Determination of sensitivity

We tested the sensitivity of RAPD markers using the artificial mixtures derived from unequal amounts of different two clones. In most cases, the markers possessed by the fewer clones were present in the bulked sample. For example, Shima-(t)64 did not possess the marker D-03 (700 bp) but Namikata-(t)73 and Tosashimizu-(t)63 did, and the marker was present in Bulk 1 and 3. Yoshida-(t)2 did not possess the marker V-17 (470 bp) but Tosashimizu-(t)63 did, and the marker was present in Bulk 6 (*Fig. 1*). Out of 20 markers, only two markers, A-19 (900 bp) and G-14 (380 bp), were absent in the bulked samples, although the fewer clones possessed these markers (*Table 3*). We excluded these markers from the rest of our study. The RAPD patterns of more than one marker in the six artificial mixtures were different from those of the single clones used for them, respectively.

Genetic identity of ramets in the seed orchard

To check the genetic identity of ramets in the seed orchard, we compared the RAPD patterns of the bulked samples (*Table 2*) with those of the standard individuals of the diagnostic clone. In most cases, the RAPD patterns of the bulked samples were consistent with those of the standard individuals. However, the RAPD patterns of three bulked samples, namely Bulk B in Namikata-(t)73, Bulk B in Namikata-(t)37, and Bulk A in Shima-(t)64, were different from those of their standard individuals, respectively. Then, we extracted DNA from a total of 12 individuals consisting of these suspicious bulked samples (4 individuals x 3 bulked samples) and analyzed them individually. For example, although the standard individual of Namikata-(t)37 didn't possess the marker U-09 (610 bp), Bulk

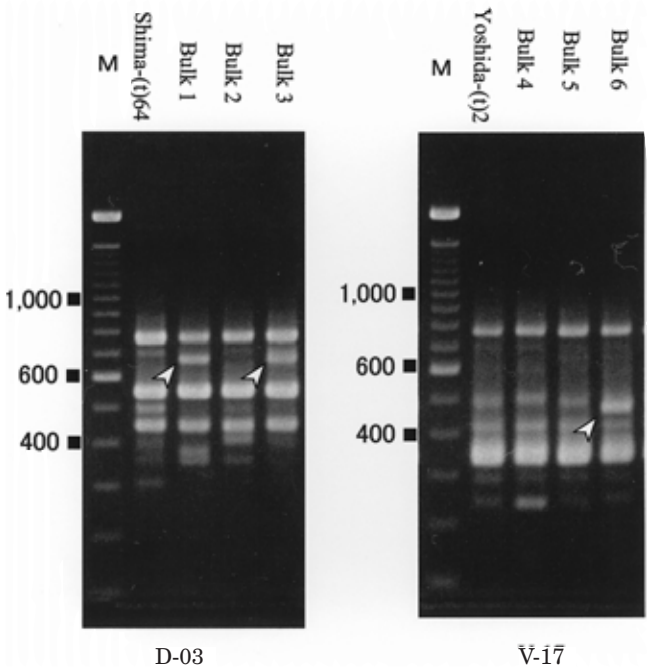

Figure 1. – RAPD patterns of the bulked samples consisting of different genotypes.
This electrophoretic profile shows a part of PCR products using RAPD primers D-03 and V-17. Lane M: 100 bp size marker (Gibco BRL), Bulk 1: Shima-(t)64 (150 mg) + Namikata-(t)73 (50 mg), Bulk 2: Shima-(t)64 (150 mg) + Obama-(t)30 (50 mg), Bulk 3: Shima-(t)64 (150 mg) + Tosashimizu-(t)63 (50 mg), Bulk 4: Yoshida-(t)2 (150 mg) + Namikata-(t)73 (50 mg), Bulk 5: Yoshida-(t)2 (150mg) + Obama-(t)30 (50 mg), Bulk 6: Yoshida-(t)2 (150 mg) + Tosashimizu-(t)63 (50 mg). *Arrows* represents RAPD markers used in this study.

Table 3. – Comparison of RAPD patterns between single clones and artificial mixtures.

Primer	bp	Shima-(t)64	Namikata-(t)73	Bulk 1	Shima-(t)64	Obama-(t)30	Bulk 2	Shima-(t)64	Tosashimizu-(t)63	Bulk 3	Yoshida-(t)2	Namikata-(t)73	Bulk 4	Yoshida-(t)2	Obama-(t)30	Bulk 5	Yoshida-(t)2	Tosashimizu-(t)63	Bulk 6
A-09	510	+	+	+	+	+	+	+	–	+	–	+	+	–	+	+	–	–	–
A-17	750	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A-19	900	–	+	+	–	–	–	–	+	– *	–	+	– *	–	–	–	–	+	– *
D-03[a]	700	–	+	+	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+
	800	+	–	+	+	+	+	+	+	+	–	–	–	–	+	+	–	+	+
F-03	720	–	+	+	–	–	–	–	+	+	–	+	+	–	–	–	–	+	+
F-05	710	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F-16	610	+	–	+	+	+	+	+	+	+	–	–	–	–	+	+	–	+	+
G-14	380	–	+	– *	–	–	–	–	–	–	–	+	– *	–	–	–	–	–	–
G-17	590	–	+	+	–	–	–	–	–	–	+	+	+	+	–	+	+	–	–
I-10	530	–	+	+	–	–	–	–	+	+	–	+	+	–	–	–	–	+	+
	750	+	+	+	+	–	+	+	–	+	+	+	+	+	–	+	+	–	+
	800	–	–	–	–	+	+	–	–	–	–	–	–	–	+	+	–	–	–
P-14	800	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	900	+	–	+	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–
S-18	700	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
U-03	700	–	+	+	–	–	–	–	+	+	–	+	+	–	–	–	–	+	+
U-09	610	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	+
U-13	580	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
V-17[b]	470	+	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–	+	+

"+" means presence, and "–" means absence. Refer Bulk 1 – 6 to *table 1.* "–*" means absence, nevertheless one of the clones possessed the fragment. The electrophoretic patterns of D-03 (700 bp)[a] and V-17 (470 bp) [b] were shown in *figure 1.*

B in Namikata-(t)37 possessed the marker, which suggested that there were any mislabeled trees within the bulked sample. As the result of individual RAPD analyses, one individual (No.139) out of four individuals in this bulked sample possessed the marker (*Fig. 2*), and we found this individual to be mislabeled. Thus, three trees, namely No. 135 from Bulk B in Namikata-(t)73, No. 139 from Bulk B in Namikata-(t)37 and No. 8 from Bulk A in Shima-(t)64, showed the different RAPD patterns from those of their standard individuals, and they were found to be mislabeled. The RAPD patterns of No. 135 was consistent with the other clone, Tosashimizu-(t)63. The RAPD patterns of No. 139 and No. 8 were not consistent with any orchard clones, and their patterns were different from each other. Therefore, these two individuals were considered to be the other genotypes that are different from the orchard clones.

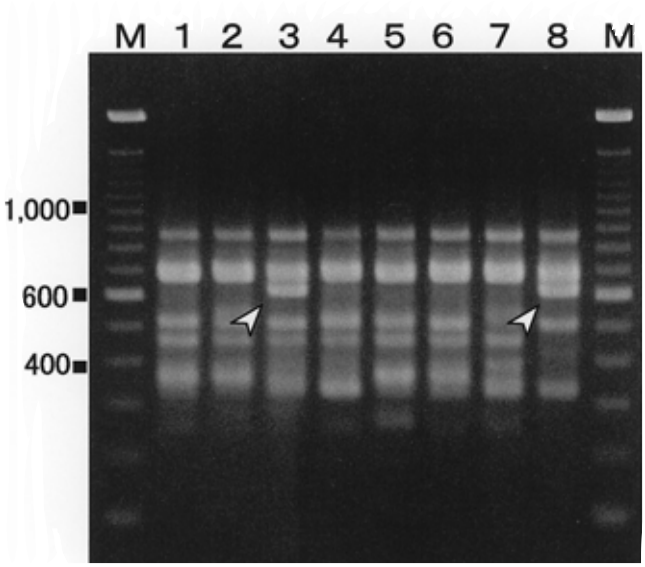

Figure 2. – Detection of mislabeled ramet in a clonal seed orchard using RAPD analysis with a bulking procedure.
This electrophoretic profile shows a part of PCR products using RAPD primer U-09. Lane M represents 100 bp size marker (Gibco BRL). Lane 1 is Namikata-(t)37 (standard individual). Lanes 2-4 represent bulked samples of Namikata-(t)37, namely lane 2: Bulk A in Namikata-(t)37, 3: Bulk B in Namikata-(t)37 and 4: Bulk C in Namikata-(t)37. Lanes 5-8 represent individuals consisting of Bulk B in Namikata-(t)37, namely lane 5: No. 58, 6: No. 85, 7: No. 112 and 8: No. 139. *Arrows* represents RAPD markers used in this study.

Discussion

The most important advantage of using a bulking procedure is the increased efficiency that it affords. In applying DNA markers to plants, the extraction of DNA is an especially laborious stage (ÅKERMAN *et al.*, 1995). To minimize this we tried to use bulked samples from several trees for RAPD analysis. In this study, we analyzed 42 bulked samples (14 clones x 3 bulked samples) and 12 individuals from the bulked samples that were suspected of being mislabeled. DNA extractions and PCR amplifications for 157 trees were replaced by those of a total of 54 samples (42 bulked samples and 12 individuals), so the workload was only about one-third of the workload when making individual RAPD analyses. It is important to check the fidelity of this bulking method. MIYAHARA *et al.* (2001) carried out individual RAPD analyses for this seed orchard after this study, and the only mislabeling they detected was the three mislabeled trees detected in this study, which demonstrated the accuracy of our method of checking bulked samples.

In this study, we mixed equal amounts of needles from a maximum of four individuals per bulked sample. The number of individuals in the bulked samples is critical when assessing the efficiency of the bulking procedure. If the number of individuals is large, the workload is lower, but in that case it will be more difficult to detect mislabeling in the bulked samples. Before beginning the present study, we mixed the needles of two different clones, which were mixed in the proportion of fourteen to one, and compared the RAPD patterns of the bulked samples with those of the single clones used for the mixtures. However, many markers were undetectable in the bulked samples (data not shown). MICHELMORE *et al.* (1991) mixed *Lactuca saligna* and *L. sativa* in the proportions 0.5, 0.4, 0.2, 0.1, 0.04, 0.02 and 0.001 and found that RAPD markers derived from less than 10% of the total DNA could not be detected in the bulked samples. YU and PAULS (1993) mixed the equal amounts of DNA samples from five individuals derived from the breeding population in alfalfa (*Medicago sativa* L.). They reported that six markers were detected in the bulked samples, out of 7 markers possessed by five individuals, but one marker was not detected. Their report was similar to ours in that most, though not all of the markers were detectable in the bulked samples. Taking previous reports and this result into consideration, we think bundling 3 to 7 individuals is reasonable for each bulked sample. The sensitivity will vary with the sequence amplified in the search for RAPD markers (YU and PAULS, 1993). This probably reflects the competition that occurs during the initial cycles of RAPD amplification between templates that mismatch to varying degrees with the primer (MICHELMORE *et al.*, 1991). In this study, two RAPD markers failed to be amplified in the bulked samples. Therefore, a pre-test of the sensitivity of RAPD markers should be carried out before the bulking procedure is extensively applied.

In seed orchard management, any errors in the genetic identity of the plant materials are clearly undesirable (WHEELER and JECH, 1992; KEIL and GRIFFIN, 1994). ADAMS (1983), using allozyme markers of both seed and needle tissues, found that 2%–13% of the ramets assayed in a Douglas-fir (*Pseudotsuga menziesii* [MIRB.] FURANCO) orchard were mislabeled. Similarly, HARJU and MUONA (1989) found 7%–10% mislabeled ramets in two Scots pine (*Pinus sylvestris* L.) orchards. In two loblolly pine (*Pinus taeda* L.) orchards, WHEELER and JECH (1992) identified mislabeled ramets in about 10% of the clones evaluated. In this study, 3 out of 157 trees (1.9%) were mislabeled. This level of mislabeling is comparable to that of previous reports. In this seed orchard, however, two mislabeled trees that are different from any orchard clones have been adult and flowered sufficiently. There were no pines observed within at least 500 meters outside from the seed orchard (MIYAHARA *et al.*, 1998), so there was a very rare chance of extra pollen. However, two mislabeled trees would be a serious source of pollen contamination to the seed orchard crops. In fact, GOTO *et al.* (unpublished) reported that some pollen contaminations were detected in the progenies from the seed orchard. VAN DE VEN and MCNICOL (1995) investigated five ramets of five clones from a Sitka spruce (*Picea sitchensis* [BONG.] CARR.) orchard and they found some trees derived from clone to be different from any orchard clones. They suggested that checking the genetic identity of ramets in a seed orchard should be carried out at the time of orchard establishment, when trees are easy to move. We support this suggestion and believe that the genetic identity of ramets in seed orchards should be checked before the plants reach adulthood.

The level of mislabeling affects the workload that we can decrease with a bulking procedure. If the level of mislabeling is high, the efficiency of this method is low. Here, we suppose the

hypothetical seed orchard that is consisting of 20 clones, 20 ramets per clone, and a total of 400 trees. If we bulked needle samples of four trees as done for this study, the workload varies depending on the level of mislabeling as follows. If 2% of trees are mislabeled, the suspicious bulked samples are maximum 8. Therefore we must analyze 100 bulked samples and 32 individual trees (4 trees x 8 suspicious bulked samples) and the workload is 33% comparing with individual RAPD analyses of 400 trees. If 5% and 10% of trees are mislabeled, the workload is similarly calculated as 45% and 65%, respectively. If 20% of trees are mislabeled, the suspicious bulked samples are maximum 80. In this case, we must analyze 100 bulked samples and 320 individuals (4 individuals x 80 suspicious bulked samples). In this case, the bulking procedure is not as efficient as individual analyses. However, the level of mislabeling in most previous reports is less than 10% (ADAMS, 1983; HARJU and MUONA, 1989; WHEELER and JECH, 1992), therefore we conclude a bulking procedure will be useful for checking the genetic identity of ramets in most clonal seed orchards.

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Literature

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New Genotypes Development of *Populus euphratica* OLIV. Using Gametoclonal Variation

By A. J. MOFIDABADI, A. JORABCHI, S. SHAHRZAD and F. MAHMODI

Research Institute of Forests and Rangelands Jaber ibnHayyan Plant Sciences Labs. P.O.Box 13185-116, Tehran, Iran

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Abstract

In order to induce genetic variation of *Populus euphratica* OLIV. through gametoclonal variation, the anthers containing microspore in tetrad-uninucleate stage were isolated and cultured in MS (MURASHIGE and SKOOG, 1962) media. Five treatments of growth regulators and two incubation conditions (light and dark) were used to induce callus from microspores. Significant differences were observed between light and dark incubation for callus induction at 0.01 level. Higher percentage of callus induction was observed in dark incubation (90%). MS medium supplemented with 2 mg/l 2,4-D and 0.5 mg/l kinetin produced most calli from microspores. The calli were subcultured for 8 months in MS medium to increase variation among regenerated plantlets. Highest plant regeneration was observed in MS medium containing 2 mg/l BA and 0.1 mg/l NAA. Five hundred and thirty-six *in vitro* plantlets were regenerated from long-term callus culture. At least 42 of the regenerants differed in some way from the original clones (7.8%). High degree of morphological variation (leaves and stems) was observed among regenerated plants. Cytological analysis on root tips of regenerated plantlets showed the presence of haploid, diploid, aneuploid and polyploid plantlets.

Key words: Populus euphratica, gametoclonal, tetrad- uninucleate, aneuploid, polyploid and haploid.