Genetic Analysis of *Eucalyptus urophylla* and *E. grandis* Clones Selected in Commercial Crops From the Brazilian Amazon by RAPD Markers

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Summary

Environmental adaptation problems and absence of flowering in *Eucalyptus grandis* populations cultivated in the Brazilian Amazon, have been solved by using hybrids of this species and *E. urophylla.* In this work RAPD molecular markers were employed to carry out genetic distance analysis of selected hybrid clones from populations S1 and S2. A total of 14 primers were used, obtaining 90 scorable bands as a product of amplification, of which 70% were polymorphic. The cluster analysis using genetic distances generated from RAPD markers grouped S2 progeny with a pattern of *E. grandis* species. The genotypes selected in S1 and S2 population formed a group with the pattern of *E. grandis* species and the *E. urophylla* species isolated. It was not possible to distinguish the hybrids that flowered from the ones that did not. However, the knowledge of genetic distances will be an important tool for choosing the appropriate genotypes for crossing in an improvement program.

Key words: DNA fingerprinting, *Eucalyptus grandis, E. urophylla,* genetic distance, RAPD markers.

Sumário

Problemas de adaptação ambiental e ausência de florescimento em populações de *Eucalyptus grandis* cultivadas na Amazônia Brasileira, tem sido solucionados com a utilização de híbridos desta espécie com *E. urophylla.* Neste trabalho marcadores moleculares RAPD foram empregados para análise da distância genética de híbridos selecionados em populações S1 e S2. Um total de 14 primers foram usados obtendo-se como produto da amplificação 90 bandas analisadas, com 70% polimórficas. A análise de agrupamento usando as distâncias genéticas geradas com marcadores moleculares RAPD agruparam as progênies S2 com um padrão para a espécie *E. grandis.* Os genótipos selecionados em S1 e S2 formaram um grupo com o padrão da espécie de *E. grandis* e o padrão da espécies *E. urophylla* ficando isolada. Não foi possível distinguir os híbridos que apresentaram florescimento daqueles que não apresentaram. Entretanto, o conhecimento das distâncias genéticas será uma importante ferramenta na escolha dos genótipos mais adequados para cruzementos dentro de um programa de melhoramento.

Introduction

Many *Eucalyptus* species have been widely used to supply the demand of wood products on the industrial Brazilian sector, mainly for pulpwood and energy production. After selecting the species and provenances which are best adapted to different environmental conditions, hybridization has been employed in order to combine desirable characteristics from different species and exploit the heterosis aiming the increase of wood production (Ferreira and Araújo, 1981; Castro, 1988).

The cultivation of *E. grandis* for its desirable characteristics has not been possible in the north of the Brazil due to adaptation problems and absence of flowers. However, *E. urophylla* and *E. grandis* hybrids have been successfully used in highly profitable plots, maintaining the technological wood qualities of *E. grandis* as well as the adaptability characteristics of *E. urophylla* (Endo and Lamberth, 1992).

The recovery of *E. grandis* characteristics by recurrent crosses and by introgression of adaptability and flowering characteristics of *E. urophylla* can lead to a breeding program of *Eucalyptus* in the Brazilian Amazon. The selection and crosses among clones of higher performance are carried out with the aim to obtain more stable and productive genotypes. However, in order to avoid losing of the genetic diversity, the crosses among divergent genotypes ought to be considered (Cruz and Regazzi, 1994).

The knowledge obtained by molecular biology has provided many tools for DNA analysis which can help in the genetic analysis of genotypes involved in breeding programs. Among these tools Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990; Welsh and McClelland, 1990) have been used for genetic analysis, as this methodology provides a rapid and simplified analysis of a large number of sampled loci all over the genome (Rafalski and Tingey, 1993).

The polymorphisms detected among genotypes, which employed these markers, when compared to pattern of amplified fragments by each primer (for binary data) establishes genetic divergence based on a vast sampling of genomes from several genotypes involved in the analysis (Jaccard, 1908; Nei and Li, 1979; Dudley, 1994).

RAPD markers have been applied to programs of genotype analysis and population of tree species (Neale et al., 1992; Grattapaglia et al., 1992; Keil and Griffin, 1994; N'goram et al., 1994; Nesbitt et al., 1995; Barril et al., 1997). In studies with natural or artificial *Eucalyptus* hybrids, the application of RAPD methodology has shown results that justify its use as an auxiliary and efficient tool (Keil and Griffin, 1994; Sale et al., 1996).

In the present study these markers were used to analyze the progeny S1 and S2 of *E. grandis* and *E. urophylla* crosses which are cultivated in the North of the Brazil. The genetic distance analysis showed the level of genetic proximity among hybrids clones and the pattern for species. The objective of this work was: To direct the improvement programs in the north of Brasil with based in genetic distance.

Material and Methods

Plant material

Twenty four samples from S1 and S2 populations from *E. grandis* and *E. urophylla* crosses were collected (*Table 1*) in the Brazilian Amazon commercial crops. The hybrids H1 to H5

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represent the S1 progeny. The hybrid H5 was autocrossed generating the other S2 hybrids, from H6 to H24 (*Table 1*). Seven samples in one mix from each of *E. grandis* and *E. urophylla* species (*Table 2*) were included in the genetic analysis.

Table 1. – Hybrids of *Eucalyptus grandis* and *E. urophylla* from commercial crops of Brazilian Amazon.

SAMPLE	FLOWERING	GENERATING	*LOCAL OF COLLETCTION
H1 – E. urograndis	absent	S1	A 121.94
H2 – E. urograndis	absent	S1	Pomar 118
H3 - E. urograndis	absent	S1	Pomar 118
H4 - E. urograndis	present	S1	Pomar 118
H5 - E. urograndis	present	S1	A 145.94
H6 - E. urograndis	absent	S2	A 145.94
H7 – E. urograndis	present	S2	A 145.94
H8 - E. urograndis	absent	S2	A 145.94
H9 – E. urograndis	absent	S2	A 145.94
H10 - E. urograndis	absent	S2	A 145.94
H11 – E. urograndis	present	S2	A 145.94
H12 - E. urograndis	absent	S2	A 145.94
H13 - E. urograndis	present	S2	A 145.94
H14 – E. urograndis	present	S2	A 145.94
H15 - E. urograndis	absent	S2	A 145.94
H16 – E. urograndis	absent	S2	A 145.94
H17 – E. urograndis	present	S2	A 145.94
H18 – E. urograndis	absent	S2	A 145.94
H19 – E. urograndis	present	S2	A 145.94
H20 – E. urograndis	present	S2	A 145.94
H21 – E. urograndis	absent	S2	A 145.94
H22 – E. urograndis	present	S2	A 145.94
H23 – E. urograndis	absent	S2	A 145.94
H24 – E. urograndis	present	S2	A 145.94

*) The samples H2, H3 and H4 were collected seed orchards and the other samples in commercial plantations.

Table 2. – Pattern of *Eucalyptus grandis* and *E. urophylla* used.

	SAMPLE	REFERENCE	LOCAL OF COLLECTION
G	01 - *E. grandis*	GRD - P	SILVICULTURA/UFV/Viçosa
	02 - *E. grandis*	GRD - 42	DENDROLOGIA/UFV/Viçosa
	03 - *E. grandis*	GRD - 43	DENDROLOGIA/UFV/Viçosa
	04 - *E. grandis*	10693	DENDROLOGIA/UFV/Viçosa
	05 - *E. grandis*	10694	DENDROLOGIA/UFV/Viçosa
	06 - *E. grandis*	10695	DENDROLOGIA/UFV/Viçosa
	07 - *E. grandis*	9783	DENDROLOGIA/UFV/Viçosa
U	08 - *E. urophylla*	URO - 12	SILVICULTURA/UFV/Viçosa
	09 - *E. urophylla*	URO - 1	DENDROLOGIA/UFV/Viçosa
	10 - *E. urophylla*	URO - 2	DENDROLOGIA/UFV/Viçosa
	11 - *E. urophylla*	URO - 3	DENDROLOGIA/UFV/Viçosa
	12 - *E. urophylla*	URO - 6	DENDROLOGIA/UFV/Viçosa
	13 - *E. urophylla*	URO - 7	DENDROLOGIA/UFV/Viçosa
	14 - *E. urophylla*	URO - 8	DENDROLOGIA/UFV/Viçosa

DNA isolation

The DNA was extracted using a method modified from that of DOYLE and DOYLE (1990), with the following modifications: 1% insoluble PVP and 0.4% β-mercaptoetanol were used in the extraction buffer. The DNA was precipitated with isopropanol and 2.5 M ammonium acetate.

Amplification of DNA fragments

The amplification reactions were done according to WILLIAMS et al. (1990) with some modifications, as following: 10 mM Tris-HCI (pH 8.3), 50 mM KCI, 2.5 mM $MgCI_2$, 0.1 mM of each deoxinucleotide (dATP, dCTP, dGTP and dTTP); 0.4 M random primer, 25 ng DNA and 1 unit of *Taq* DNA polymerase in a total volume of 25 µl. The amplifications occurred in a thermal cycler (Programmable Thermal Controller – 100 MJ Research Inc.). The thermal cycle employed was: 40 cycles of 15 sec at 94 °C, 30 sec at 35 °C and 1 min at 72 °C and 7 min at 72 °C for final extent. The temperature was decreased to 4 °C and it was maintained until the samples were removed. A total of fourteen random primers (Operon tecnologies Inc., Alameda, CA) were used in this analysis.

Amplified products were analyzed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. The bands were visualized and photographed on an ultraviolet transilluminator, using Polaroid MP-4 or Eagle Eye™ video system (Stratagene).

Data analysis

Amplified bands were scored as present (1) or absent (0) for a specific band (fragments of DNA) for different samples. The presence or absence of specific bands (similar size) in all genotypes compared shows similarity, and presence in one and absence in other shows dissimilarity.

The data were analyzed by a statistical program, GENES (CRUZ, 1997) in which the genetic distance values were calculated on the basis of the Jaccard coefficient (JACCARD, 1908), using the formula dii' = (1 – Sii') x 100, where dii' is the genetic distance among genotypes i and i'. Sii' is the similarity Jaccard coefficient Sii' = a / a+b+c, where a = bands present in two genotypes, b = bands present in genotype i, c = bands present in genotype i'. The cluster analysis was carried out using the method UPGMA (Unweighted Pair Group Method Arithmetic Average), provided by the STATISTICA version 4.2.

Results

The results of RAPD analysis are shown in *table 3*. A total of 90 bands were generated, in average 6.43 bands per primer used (varying from 3 to 10), while 63 bands were polymorphic. The amplifications with the primers: OPT03, OPT08 and OPT15 presented 100% of polymorphism. *Figure 1* shows the amplification pattern obtained with primer OPB 12.

Table 3. – Primers used in RAPD amplification, sequences, number of analyzed bands, number of polymorphic bands, percentage of polymorphism.

Primers	Sequence (5' → 3')	Analyzed fragments	Polymorphic fragments	% of polymorphism
OPB 10	CTGCTGGGAC	6	3	50
OPB12	CCTTGACGCA	10	8	80
OPB16	TTTGCCCGGA	9	7	77,8
OPJ 12	GTCCCGTGGT	8	7	87,5
OPJ 15	TGTAGCAGGG	8	4	50
OPJ 19	GGACACCACT	6	2	33,3
OPT 01	GGGCCACTCA	8	5	62,5
OPT 02	GGAGAGACTC	6	5	83,3
OPT 03	TCCACTCCTG	5	5	100
OPT 04	CACAGAGGGA	4	3	75
OPT 08	AACGGCGACA	5	5	100
OPT 14	AATGCCGCAG	3	2	66,7
OPT 15	GGATGCCACT	3	3	100
OPT16	GGTGAACGCT	9	4	44,4
Total = 14		90	63	70

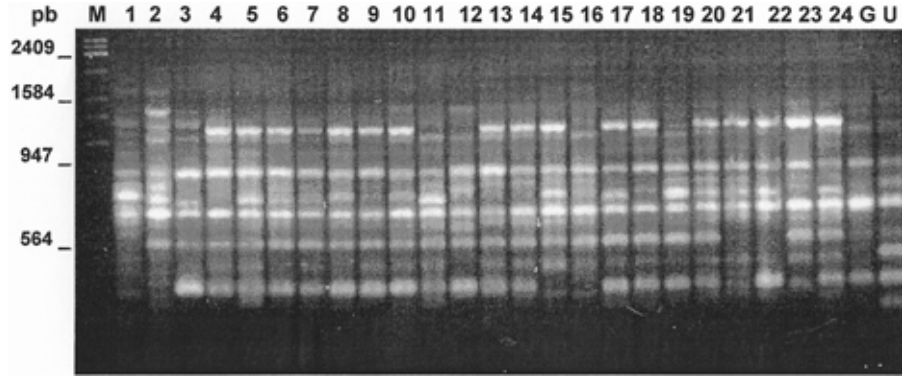

Fig. 1. – Gel electrophoresis of random amplified polymorphic DNA (RAPD) fragments obtained with primer OPB 12. Samples are identified in *table 1* and *2.* Fragment size markers in base pairs are indicated by M (Lambda digested with *Eco RI, Bam HI* e *Hind III*).

The genetic distances between the analyzed individuals were calculated according to JACCARD method to obtain a matrix of genetic distance (*Table 4*) where the smaller genetic distance was between individuals G and H3, H24 and H23, H24 and H14 (7%), and the greater distance and occurred between H16 and the pattern of *E. urophylla* species (31%). The greater genetic distance did not occur between the two pattern of the species, as expected. The genetic distance among the *E. grandis* pattern and the other hybrids were less than 20%, and the distance between *E. urophylla* pattern and the hybrids were similar or greater than this value.

The matrix of genetic distance was used to generate a dendrogram (*Fig. 2*) in which the genotypes were grouped by UPGMA method, where each group was formed by individuals of greater similarity. The greater genetic distance value between two individuals according to cluster analysis indicates that they are very divergent based on the mean distance related to the other individuals involved in the analysis.

The dendrogram shows groups of hybrids with the *E. grandis* pattern within a genetic distance smaller than 23.5% and also a clear separation from the pattern of *E. urophylla* species.

When the individual components of the DNA mix were analyzed separately, a high homogeneity was observed among the individuals of the same species and a high heterogeneity between individuals of the two species. The average genetic distance within *E. grandis* was 22%, and for *E. urophylla* was 11% (date not shown). Competition may happen for some of the RAPD fragments in the mixture, however the fragments that are amplified are generally present in most of the individuals of the mix (*Figure 3*). This allows a comparison of the clones selected with markers that are represented in larger percentage in the species.

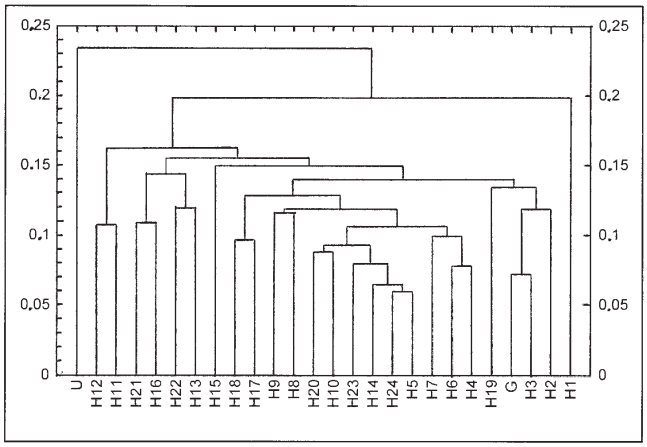

Fig. 2. – UPGMA cluster diagram of relationships among hybrids of *Eucalyptus grandis, E. urophylla* and pattern of these species. The dendrogram was obtained by cluster analysis of genetic distance values calculated from RAPD data obtained by using 14 different RAPD primers. (G) *Eucalyptus grandis,* (U) *E. urophylla*, (Hn) hybrids. Samples are identified in *table 1.*

Table 4. – Pairwise genetic distances among hybrids of *Eucalyptus grandis* and *E. urophylla*, and pattern of these species. Samples are identified in *tables 1* and *2.*

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	G
H2	0,13	0,00																							
H3	0,20	0,12	0,00																						
H4	0,17	0,15	0,13	0,00																					
H5	0,22	0,16	0,15	0,09	0,00																				
H6	0,20	0,15	0,15	0,08	0,09	0,00																			
H7	0,18	0,14	0,17	0,09	0,09	0,11	0,00																		
H8	0,21	0,18	0,16	0,13	0,13	0,09	0,11	0,00																	
H9	0,18	0,18	0,18	0,13	0,10	0,13	0,12	0,12	0,00																
H10	0,17	0,12	0,15	0,09	0,10	0,10	0,10	0,11	0,13	0,00															
H11	0,21	0,15	0,17	0,12	0,11	0,12	0,12	0,13	0,13	0,12	0,00														
H12	0,21	0,17	0,21	0,18	0,18	0,20	0,19	0,22	0,18	0,18	0,11	0,00													
H13	0,20	0,21	0,23	0,12	0,11	0,11	0,14	0,13	0,15	0,14	0,18	0,20	0,00												
H14	0,21	0,14	0,14	0,11	0,06	0,11	0,11	0,12	0,09	0,08	0,11	0,14	0,12	0,00											
H15	0,20	0,18	0,15	0,13	0,13	0,13	0,14	0,14	0,15	0,16	0,17	0,24	0,20	0,15	0,00										
H16	0,19	0,19	0,18	0,16	0,12	0,15	0,14	0,15	0,17	0,18	0,13	0,15	0,13	0,15	0,18	0,00									
H17	0,22	0,18	0,13	0,12	0,07	0,12	0,10	0,14	0,13	0,13	0,10	0,20	0,19	0,11	0,13	0,13	0,00								
H18	0,20	0,16	0,14	0,11	0,13	0,14	0,15	0,14	0,21	0,12	0,15	0,24	0,22	0,16	0,16	0,20	0,10	0,00							
H19	0,18	0,15	0,14	0,12	0,13	0,11	0,14	0,13	0,17	0,12	0,12	0,19	0,17	0,15	0,18	0,13	0,14	0,17	0,00						
H20	0,19	0,15	0,11	0,10	0,08	0,11	0,15	0,14	0,14	0,09	0,11	0,18	0,15	0,09	0,14	0,15	0,10	0,09	0,13	0,00					
H21	0,24	0,21	0,21	0,13	0,08	0,13	0,13	0,17	0,18	0,18	0,15	0,19	0,14	0,15	0,17	0,11	0,13	0,20	0,15	0,13	0,00				
H22	0,27	0,21	0,19	0,13	0,10	0,11	0,17	0,16	0,18	0,16	0,16	0,23	0,12	0,12	0,21	0,16	0,15	0,16	0,18	0,14	0,14	0,00			
H23	0,20	0,13	0,15	0,13	0,08	0,11	0,13	0,13	0,13	0,12	0,16	0,21	0,13	0,09	0,17	0,13	0,10	0,17	0,15	0,11	0,16	0,12	0,00		
H24	0,22	0,13	0,13	0,11	0,06	0,10	0,09	0,11	0,10	0,10	0,08	0,13	0,12	0,07	0,13	0,12	0,11	0,18	0,11	0,08	0,11	0,13	0,07	0,00	
G	0,18	0,12	0,07	0,12	0,13	0,12	0,14	0,16	0,16	0,10	0,13	0,18	0,19	0,12	0,17	0,15	0,12	0,15	0,11	0,11	0,18	0,16	0,13	0,11	0,00
U	0,23	0,20	0,21	0,22	0,22	0,23	0,21	0,23	0,20	0,21	0,23	0,24	0,27	0,22	0,26	0,31	0,23	0,22	0,21	0,25	0,29	0,28	0,24	0,23	0,20

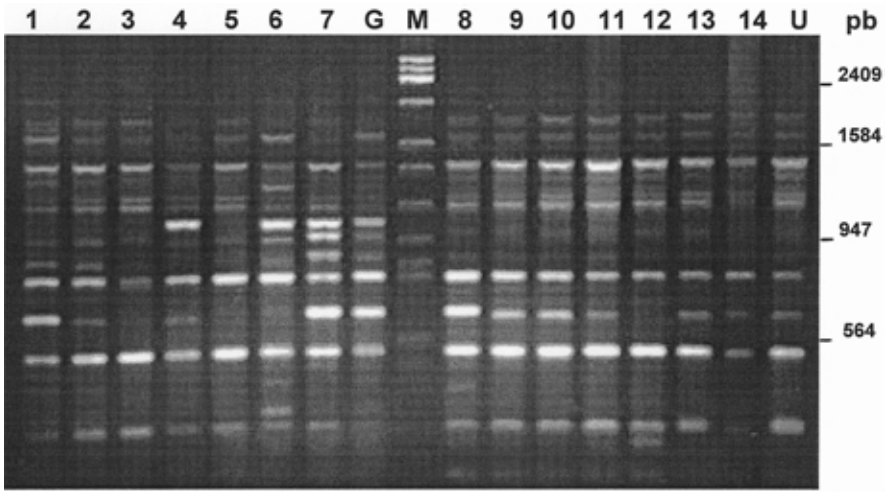

Fig. 3. – Gel electrophoresis of random amplified polymorphic DNA (RAPD) fragments obtained with primer OPJ 19. Samples are identified in *table 2.* Fragment size markers in base pairs are indicated by M (Lambda digested with *Eco RI, Bam HI* e *Hind III*).

Considering distances inferior to 15% it was possible to observe the formation of three groups and the separation of hybrid H1. The first group is formed by H11 and H12, the second H13, H16, H21 and H22 and the third is formed by the other hybrids.

Discussion

The hybrids used in these analyses are part of na improvement program, and resulted from the selection in the S1 and S2 populations for characteristic of the *E. grandis* species. Therefore one reason for the separation of the *E. urophylla* pattern from the other hybrids may be related to the selection technique. Taking into account that *E. grandis* species present greater production values in its harvesting region, the pressure on selection benefited the similar genotypes of *E. grandis* species, approaching the selected hybrids in S1 to H5 progenies. Concomitantly there would be a decrease of genes contribution of *E. urophylla* in the selected clones.

The hybrid clone H5, which expectedly autocrossed, presented smaller genetic distance in relation to its progenies (H6 to H24), as shown in the dendrogram and matrix (*Fig. 2* and *Table 4*).

The hybrids H1, H2, H3, and H4 belong to S1 progeny. H1 and H4 are phenotypically similar to *E. urophylla* and H2 and H3 are similar to *E. grandis* pattern. The dendrogram shows a separation of H1, and H2 / H3 grouping with *E. grandis* pattern, confirming, in part, the phenotypical evaluation from the field. However, H4 grouped to *E. grandis* pattern. Studies of populations of natural hybrids using RAPD markers suggested that morphological analysis can not necessarily point out the genetic contribution of both parental species (SALE et al., 1996).

Among S2 genotypes, which presented smaller distance from *E. grandis* pattern (11%), the hybrids H19, H20 and H24 maintained the flowering characteristics. Despite the fact that H10 presented the smallest distance (10%) it did not flower. These data show the possibility of using RAPD markers to help with autocross programs, recuperating desirable characteristics from one of the progenitors keeping the desirable characteristics of the other parental.

In the present research, based on molecular analysis, we could not verify groupings of individuals, that corresponds to the phenological differences with presence or absence of flowering. This is a very important characteristic because *E. grandis* did not flower in Jari region, but only in the South of the country due to genotype-environmental interaction.

The phenological behavior of individuals from S1 and S2 populations showed the occurrence of segregation for flowering characteristics. The genetic distance based on RAPD markers, instituted from a immense sample in the genome, is a complex characteristic.

The analysis of genetic distances based on RAPD markers allowed us to determine the genetic distance between hybrid clones and between them and their parents. It helps direct the breeding program on choosing genotypes more divergent for simple crosses denominated Negative Associating Cross or for Positive Associating Crosses, employing hybrids progenies which are closer. Because of the superiority of *E. grandis* for pulpwood RAPD markers can be used to efficiently direct the recovery of species characters from a cross.

It was not possible to distinguish individuals that were flower present or absent by using genetic distances obtained from these markers, since flowering control is very complex. The clone H5 flowered and its S2 produced individuals that were flower present and absent. This segregation indicates a recombination genes which control this characteristic.

The characterization and flowering control analysis require knowledge of specific regions of the genome that contribute for this control, consisting of an efficient characterization of parents, controlled crosses and finally analysis of segregating progenies (S2) formed by a considerable amount of individuals, for genetic mapping, and detection of a possible QTL for this characteristic.

The knowledge of genetic structure of the population involved in a breeding program is the first step to define strategies to the selection or/and the crosses to be employed, RAPD markers showed as an important tool on genetic analysis of population and genotypes, mainly in forestry activities, where the long period to generate species demand more efficient and quicker analysis.

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The New OECD Scheme for the Certification of Forest Reproductive Materials

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Summary

The old 1974 OECD Scheme has been updated by 1995/96 in order to include new progress in forest tree improvement (OECD, 1997).

Types of Basic Materials are now: Seed Sources, Stands, Seed plantations, Seed orchards, Parents of families, Clones and Clonal mixtures.

Categories of Forest Reproductive Materials produced by these Basic Materials are similarly now: Source Identified (yellow tag), Selected (green), Qualified (pink) and Tested (blue).

This System is explained in simple terms and the synthesis is done by *Table 1*.

Recommendations are given to apply the Scheme as clearly and simply as possible. They are mainly relevant to the structure and subdivisions of the National Catalogue, information to insert in this catalogue and the use of colours.

Though foreseen for international trade, this system can be used within countries as well. Procedure to join the OECD Scheme is also briefly outlined.

Hopefully, the new European Directive should stay fully compatible with the OECD Scheme.

OECD Scheme is also considered as a powerful tool for canalising research, forest tree improvement and conservation programmes into an integrated and efficient global strategy with use of tree seed centres for afforestation programmes.

Varietal outputs of these programmes, themselves commercially produced by national tree seed centres and certified according to the OECD Scheme should permit to realise the large afforestations of high quality that are requested by the great economical and ecological challenges of the XXI th century.

Key words: OECD, certification, Forest Reproductive Materials, Basic Materials, EU Directive, forest tree improvement and research, genetic conservation, tree seed centres, afforestation.

Résumé

L'ancien système de l'OCDE de 1974 a été mis à jour en 1995/96, de façon à intégrer les nouveaux progrès en amélioration des arbres forestiers (OCDE, 1997).

Les Types de Matériels de Base sont maintenant: les Sources de graines, les Peuplements, les Plantations issues de graines, les Vergers à graines, les Parents de familles, les Clones et les Mélanges de clones.

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