controlled in the immediate future. If the work being done by TAFORI in collaboration with the International Institute of Biological Control (IIBC) gives immediate results for the biological control of *C. cupressi* a revisit of these recommendations is imperative.

Conclusion and Recommendations

Results from this study call for a qualified set of recommendations concerning suitable families that merit further breeding work.

Based exclusively on the results from this trial, suitable families 'judged on the basis of high volume production and resistance to *Cinara* infestation' that merit further breeding work are K9, K152, and K157. When the results are combined with those from another study (OBIRI *et al*., 1994) involving more sites, families U1, U3, K152, K150 and K157 merit further breeding work. These recommendations pre-suppose slow advances in the biological control of the *C. cupressi* infestation.

When *C. cupressi* is finally contained, the best 10 families identified in this study namely: K9, U6, K152, U1, U3, K87, K150, K157, K160, and K162 merit further breeding work. This large number of families will widen the genetic base.

As family representation does not involve equal numbers from Kenya, Uganda and Tanzania the conclusions given call for further caution.

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Isozyme Characterization of *Eucalyptus urophylla* (S. T. BLAKE) and *E. grandis* (HILL ex MAIDEN) Populations in Brazil

By M. P. MARTINS-CORDER[1]) and C. R. LOPES

Department of Genetics, Biosciences Institute, UNESP, Botucatu, São Paulo, Brazil

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Abstract

Seedling taken from 2 species of *Eucalyptus* growing in Brazil were electrophoretically analysed at 14 isozyme loci representing 6 enzyme systems: α-EST, ß-EST, SKDH, IDH, MDH, and LAP. Genetic variability measures were determined using 11 putative isozyme loci. On average, 81.8% and 54.5% of the loci were found to be polymorphic by the criterion of 95% in *E. urophylla* and *E. grandis*, respectively. The mean number of alleles per loci was 3.0 in *E. urophylla* and 2.5 in *E. grandis*. Observed mean heterozygosity was 0.283 in *E. urophylla* and 0.166 in *E. grandis*. Levels of genetic diversity in these species were similar to those in other *Eucalyptus* species which have

[1]) Present address: Departamento de Ciências Florestais, Centro de Ciências Rurais, Universidade Federal de Santa Maria, 97117-900 Santa Maria, Rio Grande do Sul, Brazil

widespread distributions. The possible hybridization of *E. urophylla* with *E. alba* is also discussed.

Key words: *Eucalyptus urophylla*, *E. grandis*, *E. alba*, polymorphism, electrophoresis, genetic diversity.

FDC: 165.3; 165.5; 176.1 *Eucalyptus urophylla*; 176.1 *Eucalyptus grandis*; (81).

Introduction

Eucalyptus is a woody genus extensively planted throughout the world, especially in the tropics (FAO, 1982). In Brazil, more than 3 000 000 ha are planted with several well know *Eucalyptus* species (GARCIA and PIMENTEL-GOMES, 1992). The edaphic and climatic conditions of Brazil are excellent for cultivation of eucalyptus, with a wood productivity of 40 m^3/ha/year to 50 m^3/ha/year and a rotation cycle of 6 to 8 years.

Eucalyptus grandis (HILL ex MAIDEN) and *E. urophylla* (S.T. BLAKE) are among the species most frequently planted in Brazil because of their excellent performance. Their wood is appropriate for several industrial uses such as the manufacture of paper and cellulose, timber and poles.

An understanding of the genetic structure of any species is essential for the optimal utilization of genetic resources in terms of both genetic improvement and conservation. Information on level of allozyme diversity can be very useful for determining on which species and provenances to begin genetic improvement. It is of paramount importance to evaluate and maintain genetic variability within the stands because plantations consisting of genetically uniform material may be vulnerable to major climate flutuations and pests (ARADHYA and PHILLIPS, 1993). When interest is focussed on the adaptative effects of the detected variation, that is, in the relationship between the genetic base and the phenotypic expression, as it occurs in the breeding and conservation studies, the enzyme polymorphisms are closer to the final phenotypic expression, because they are an intermediate product of the genic expression. Although then is no conclusive evidence which shows significant correlations between allozyme variation distribution and the quantitative traits (HAMRICK and GOOT, 1990), the role of the isozymes as genetic markers is of great importance in plant breeding and for the conservation of genetic resources (ADAMS, 1983; BROWN and MORAN, 1979; CHELIAK *et al*., 1987; TSAFTARIS, 1987).

Isozymes have proven to be the most efficient and inexpensive method for the study of genetic variation in tree species when compared to other molecular markers or morphological characteristics (YEH, 1989). A number of studies has been published on isozyme variation in the genus *Eucalyptus* (ARADHYA and PHILLIPS, 1993; BROWN *et al*., 1975; BURGESS and BELL, 1983; BURGESS *et al*., 1985; FRIPP, 1982; HOPPER and MORAN, 1981; HOUSE and BELL, 1994; MORAN and BELL, 1983; MORAN and HOPPER, 1983; YEH *et al*., 1983).

This study has analysed 11 putative isozyme loci in *E. grandis* and *E. urophylla* from a Seed Production Area in Brazil. The aim was to investigate variability within these species which have high priority in breeding programs in Brazil.

Material and Methods

The 2 populations used in this study and their major characteristics are listed in *table 1*. The material was collected in Seed Production Areas where mixtures of seeds from several trees per stand were obtained. Seedlings were produced in plastic tubes containing an appropriate substrate (50% soil and 50% vermiculite) and raised under nursery conditions in half shade. The seedlings were submitted to daily irrigation and weekly fertilization with nitrogen compounds with the objective of producing tender leaf tissues.

Electrophoresis

Juvenile leaves from seedlings about 6 months old were used for electrophoretic analysis. A total of 300 *E. grandis* individuals and 300 *E. urophylla* individuals were analyzed.

Approximately 400 mg of leaves were macerated in 200 µl NAMKOONG extracting solution in porcelain plates over ice by the method of by ALFENAS *et al*. (1991). The composition of the solution is 0.034 M disodium phosphate, 0.2 M sucrose, 2.56% polyvinylpyrrolidone (PVP-40), 3 mM dithiothreitol (DTT), 5.7 mM L-ascorbic acid, 5.8 mM diethyldithiocarbamic acid (DIECA), 2.6 mM sodium bisulfite, 2.5 mM sodium borate, 0.2% 2-mercaptoethanol, 1% polyethylene glycol 8000, and 100 ml deionized (or distilled) water. During trituration, a trace amount of polyvinylpolypyrrolidone (PVPP) was added to remove phenol compounds and to increase enzyme stability. The resulting slurry was absorbed onto Whatman #3 filter paper wicks (6 mm x 12 mm) and those were loaded into gel cornstarch. The gels were previously prepared with 13% cornstarch according to the procedure of CONKLE *et al*. (1982).

Electrophoresis was conducted in a refrigerator at 4 °C and 200 volts with 20 milliamperes for 8 hours (buffer system TC) and 130 volts with 25 milliamperes for 10 hours (buffer system CM) in presented *table 2*. At the end of the electrophoresis, the gels were sliced horizontally into 4 laminae, stained, and assayed for 3 enzyme systems respectively. Each seedling was analyzed for electrophoretic variants in the 6 enzyme systems: α-Esterase, β-Esterase, Isocitrate dehydrogenase, Leucine aminopeptidase, Malate dehydrogenase e Shikimate dehydrogenase.

The genetic control of the isozymes was inferred by reference to the banding patterns obtained in previous studies (MORAN

Table 1. – Details of the Brazilian *Eucalyptus* spp. populations used in this study.

Species	Origin	Provenance and lot	Latitude (N)	Longitude (W)	Altitude (m)	Number of mothers tree
E.grandis	Coff's Harbour (Australia)	Salto - SP (Brazil) G-140	21°20'	45°23'	620	25
E.urophylla	Dilli (Timor ex Portugues)	Lençóis Paulista (Brazil) U-096	22°36'	48°48'	560	30

Table 2. – The stained enzymes and their abbreviations, number of putative loci, EC numbers, gel buffer systems, and references for the staining method.

Enzyme name and abbreviation	Number of locus	EC nº.	Buffer system (1)	Reference
α-Esterase (α-EST)	5	EC 3.1.1.1	TC	Stuber *et al.* (1983)
ß-Esterase (ß-EST)	3	EC 3.1.1.1	TC	Stuber *et al.* (1983)
Isocitrate dehydrogenase (IDH)	1	EC1.1.1.42	CM	Soltis *et al.* (1983)
Leucine aminopeptidase (LAP)	2	EC3.4.11.1	CM	Soltis *et al.* (1983)
Malate dehydrogenase (MDH)	2	EC1.1.1.37	CM	Yamada & Guries (1989)
Shikimate dehydrogenase (SKDH)	1	EC1.1.1.25	TC	Yamada & Guries (1989)

(1) The buffer systems employed were: TC: electrode, 0.223 M Tris + 0.086 M citric acid adjusted to pH 7.5 with Tris; gel, 1:35 dilution of the electrode buffer (SOLTIS *et al.*, 1983); CM: electrode, 0.04 M citric acid adjusted to pH 6.1 with N-(3-aminopropyl)morpholine; gel, 1:20 dilution of the electrode buffer (CLAYTON and TRETIAK, 1972).

and BELL, 1983; MARTINS-CORDER, 1993). For each enzyme system, the isozyme of most anodal migration was designated as locus 1, the next as locus 2, and so forth. Similarly, at each locus, the band of fastest migration was designated allele 1, and progressively slower bands were designated 2, 3 etc.

Data Analysis

The Biosys computer program (SWOFFORD and SELANDER, 1989) was used to analyze the isozyme data for allele frequencies, percent polymorphic loci, mean number of alleles per locus, estimated observed and expected heterozygosity, fixation index, F statistic and chi-square contingency test.

Genetic variability was measured on the basis of 11 isozyme loci in the *E. grandis* and *E. urophylla* populations.

Results and Discussion

For the estimates of genetic variability the frequencies of 33 allelic bands for *E. urophylla* and 28 allelic bands for *E. grandis* at 11 putative loci were utilized (*Table 3*).

The α-Est and ß-Est presented 5 and 3 putative loci, respectively, with the first putative loci in each being quite similar in terms of mobility (Rf). Consequently, the ß-Est loci were not included in the statistical analysis. The Mdh-1, α-Est-4 and α-Est-5 were monomorphic in *E. grandis*, and in *E. urophylla* Mdh-1 was constant. The other isozyme loci were polymorphic in all the populations. The most common alleles were the same in approximately 82% of the total number of loci analyzed. Differences were detected in Skdh, in which the most common allele in *E. urophylla* was allele 2 while in *E. grandis* was allele 1 and in Lap-2 the most common allele in *E. grandis* was allele 3, whereas in *E. urophylla* was allele 1. The contingency test detected significant differences in allelic frequencies between species at most of the other loci analyzed (*Table 3*).

Genetic variability measured within populations is presented in *table 4*. The mean number of alleles in *E. urophylla* was about 17% higher than in *E. grandis* and the mean percentage of polymorphic loci was also higher in *E. urophylla* than in *E. grandis*. There was a larger number of alleles per locus in *E. urophylla*, for loci α-Est-4, α-Est-5 and Skdh, with 2 more alleles in each locus. In *E. grandis,* α-Est-3 had a third allele, although at low frequency of 0.2%. Loci α-Est-4 and α-Est-5 showed unique behaviors because in the *E. grandis* population, only allele 1 appeared, so that heterozygosity values were null at any one of these loci. In contrast, new allelic bands appeared at each of these putative loci in *E. urophylla* (*Table 3*). Rare alleles with frequencies less than 0.005, were detected at the α-Est-3, Skdh and Idh loci.

Heterozygosity measured at the level of loci ranged between 0 and 0.645 in *E. urophylla* and 0 and 0.453 in *E. grandis*. In general, heterozygosity at the locus level was higher in *E. urophylla* than in *E. grandis*. Genetic variability was found to be high in the populations studied and the same alleles tended to be distributed randomly within the populations of species studied.

Observed (Ho) and expected (He) heterozygosity ranged from 0.283 to 0.343 in *E. urophylla* and from 0.166 to 0.223 in *E. grandis* (*Table 4*). The highest values were for Lap-1 and the lowest for α-Est-3 in both species. The heterozygosity value of a locus increases according to the number of alleles in segregation and with the closer proximity of allele frequencies. Expected heterozygosity at the locus level was usually higher than observed heterozygosity, thus leading to a considerable deficiency of heterozygotes. Means F values ranged from 0.159 (*E. urophylla*) to 0.261 (*E. grandis*).

The results showed that is occuring considerably deviations from panmixia. The high rate of selfing, measured by the high fixation index, may possibly be the major factor responsible for

Table 3. – Allele frequency, sample size (N), mean heterozygosity per locus (H) and contingency test for chi-square heterogeneity (x^2) in 2 populations of *Eucalyptus* spp, on the basis of 11 putative loci.

Locus	Population	Designation and allele frequency					Sample size (N)	H	X^2
		1	2	3	4	5			
α-Est-1	*E.urophylla*	0.439	0.043	0.204	0.313		230	0.400	172.010(3)**
	E.grandis	0.796	0.013	0.148	0.043		270	0.263	
α-Est-2	*E.urophylla*	0.898	0.096	0.060			235	0.162	11.866(2)*
	E.grandis	0.954	0.042	0.040	0.018		271	0.063	
α-Est-3	*E.urophylla*	0.979	0.021				258	0.043	1.50(2) ns
	E.grandis	0.974	0.024	0.002			270	0.044	
α-Est-4	*E.urophylla*	0.690	0.198	0.112			237	0.384	195.863(2)**
	E.grandis	1.000					270	0.000	
α-Est-5	*E.urophylla*	0.871	0.086	0.043			93	0.151	67.115(2)**
	E.grandis	1.000					251	0.000	
Skdh	*E.urophylla*	0.348	0.424	0.004	0.184	0.040	237	0.553	341.106(4)**
	E.grandis	0.890	0.032		0.078		268	0.164	
Mdh-1	*E.urophylla*	1.000					259	0.000	
	E.grandis	1.000					268	0.000	
Mdh-2	*E.urophylla*	0.907	0.093				259	0.170	31.482(1)**
	E.grandis	0.782	0.218				268	0.354	
Idh	*E.urophylla*	0.920	0.078	0.002			257	0.097	12.587(2)**
	E.grandis	0.850	0.146	0.004			267	0.037	
Lap-1	*E.urophylla*	0.521	0.020	0.074	0.385		256	0.645	175.026(3)**
	E.grandis	0.665	0.122	0.144	0.069		267	0.453	
Lap-2	*E.urophylla*	0.509	0.252	0.239			224	0.509	9.552(2)**
	E.grandis	0.333	0.246	0.420			264	0.443	
TOTAL									**1017.654****

The numbers in parentheses correspond to the degrees of freedom.
ns no significant
**) significant at the 1% level of probability
*) significant at the 5% level of probability.

the deviations in the HARDY-WEINBERG equilibrium in these *Eucalyptus* populations.

The overall mean values obtained for percent polymorphic loci, number of alleles per locus, and mean heterozygosity, were higher in *E. urophylla* than in *E. grandis*. Compared to studies of ARADHYA and PHILLIPS (1993), and HOUSE and BELL (1994) which included *E. grandis* and *E. urophylla* populations, the present study showed that the populations have similar levels of genetic diversity.

However, these comparisons always have a high margin of error and should therefore be considered with caution. It is essential that the results include a large number of loci, preferably the same, to safely compare different species. According to GURIES and LEDIG (1982), the variation of levels of heterozygosity that exists among woody species is probably a response to the considerable temporal and spatial heterogeneity experienced by long-lived perennial species of wide distribution.

The *E. urophylla* population originated from only 2 trees, as indicated in the records of the introduction of the species to Brazil. Gene exchanges (hybridization) with *E. alba* may have occurred (M. FERREIRA, personal communication) since these exchanges occur naturally at an altitude (1240 m) similar to that where *E. urophylla* seeds were collected. Thus, it is possible that the material introduced to Brazil for seed production purposes was of hybrid origin. The descendants which were analyzed in the present study belonged to segregant generations. The individuals with undesirable characteristics (of trunk, height etc.), typical of *E. alba*, were probably eliminated by thinning, so that only trees phenotypically more similar to *E. urophylla* remained for seed production. There is some evidence based on informal reports that this genetic

Table 4. – Isozyme variability in *E. urophylla* and *E. grandis* determined on the basis of 11 putative loci.

Population	Mean sample size/ locus (N)	Mean number of alleles/locus (Ap)	Percent polymorphic loci (P %)	Mean heterozygosity Ho	He
E.urophylla	231.4	3.0	81.8	0.283	0.343
	(14.4)	(0.3)		(0.067)	(0.078)
E.grandis	266.7	2.5	54.5	0.166	0.223
	(1.7)	(0.3)		(0.055)	(0.067)

The numbers in parentheses correspond to the standard deviation.
A locus is considered polymorphic when the frequency of the most common allele does not exceed a value of 0.95.

Table 5. – Observed (Ho) and expected (He) heterozygote frequency and fixation index (F) at 10 loci of *Eucalyptus urophylla* and 8 loci of *Eucalyptus grandis*.

Locus	Population	Ho	He	F
α-Est-1	*E.urophylla*	92	153.403	0.399
	E.grandis	71	92.506	0.231
α-Est-2	*E.urophylla*	38	43.976	0.124
	E.grandis	17	23.976	0.290
α-Est-3	*E.urophylla*	11	10.789	-0.022
	E.grandis	12	13.686	0.122
α-Est-4	*E.urophylla*	91	112.159	0.187
	E.grandis			
α-Est-5	*E.urophylla*	14	21.708	0.352
	E.grandis			
Skdh	*E.urophylla*	131	157.628	0.167
	E.grandis	44	53.938	0.183
Mdh-2	*E.urophylla*	44	43.636	-0.010
	E.grandis	95	91.632	-0.039
Idh	*E.urophylla*	25	37.881	0.339
	E.grandis	10	68.435	0.854
Lap-1	*E.urophylla*	165	147.262	-0.123
	E.grandis	121	138.469	0.125
Lap-2	*E.urophylla*	114	139.264	0.180
	E.grandis	117	172.319	0.320
Mean	*E.urophylla*	72.5	86.721	0.159
	E.grandis	60.9	81.870	0.261
Overal mean		66.7	84.296	0.210

material may have produced an unsatisfactory performance with respect to tree form also in other regions of Brazil.

Consequently, it would be risky to continue a program of genetic breeding using the *E. urophylla* population, as this species may be considered not to be pure. Furthermore, the population originated from only 2 trees, a fact that would certainly lead to inbreeding depression within a short period of time.

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Stone Pine (*Pinus cembra* L.) Provenance Experiment in Romania

I. Nursery Stage at Age 6 [1])

By I. BLADA

Forest Research Institute, Sos. Stefanesti 128, 72904 Bucharest 11, Romania

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Summary

The paper reports results of a stone pine (*Pinus cembra* L.) provenance study conducted in a 4-replicated complete block design nursery provenance test. At age 6, total height growth, height increment in the 6-th year, diameter at root-collar and number of buds around the leader bud were statistically analysed. Highly significant ($p < 0.001$) differences between provenances were noticed for all tested traits. DUNCAN's test suggested a major gap between genetically isolated populations within the natural range of the species. After 6 years of testing, one can not conclude whether the species is characterized by a continuous or a discontinuous distribution pattern. Fast growing and slow growing provenances were found in populations in both the Alps and Carpathians, suggesting that the improvement by selection would be profitable.

Key words: *Pinus cembra*, population, provenance, variation, correlation.

FDC: 165.5; 232.1; 174.7 *Pinus cembra*; (498).

Introduction

The natural distribution of the stone pine (*Pinus cembra* L.) is restricted to the high altitudes of the Alps and Carpathians (SOMORA, 1959; HOLZER, 1963; CRITCHFIELD and LITTLE, 1963). In the Alps, the species ranges between 1200 m and 2500 m elevation (CONTINI and LAVARELO, 1982) but the main zone is between 1500 m and 2000 m (HOLZER, 1975). In Romania, stone pine ranges between 1350 m and 1880 m elevation in the northern Carpathians (GUBESH, 1971) and between 1350 m and 1986 m in the southern Carpathians (BELDIE, 1941; TATARANU and COSTEA, 1952; OARCEA, 1966).

The stone pine is important for:

– reforestation of the subalpine zone to raise the timberline, where it plays a leading part in watersheds, for stabilizing avalanche areas and for reducing the effects of the flash floods (HOLZER, 1972, 1975);

– spruce – larch – cembra mixed stands creation at high elevation in order to increase their windbreak resistance (BLADA, 1996);

–its dense – brown – reddish wood useful for handicrafts (CONTINI and LAVARELO, 1982);

– its high resistance to blister – rust caused by *Cronartium ribicola* FISCH. ex. RABENH. (BINGHAM, 1972; HOLZER, 1975; HOFF et al., 1980; BLADA, 1982, 1987, 1990, 1994);

– landscaping purposes due to its conic – oval shape when grown as single tree (BLADA, 1996).

Investigations concerning stone pine variability revealed that:

– a phenotypical variability in crown shape and stem form was noticed (RIKLI, 1909; HOLZER, 1975);

– in 2 trials with half-sib progenies, HOLZER (1975) found a good correlation between height growth and the elevation of the seed source, i.e. at low altitudes the provenances from the lowest elevation grew best and the progenies of trees selected near the timber line grew better at higher elevations;

– very high variation in both the number of seeds per cone and weight of seeds per cone was found within each investigated population, but variation in 1000 seed weight was moderate (BLADA and POPESCU, 1992);

– highly significant differences between provenances for growth traits were found (BLADA, 1987);

– the *P. cembra* x *P. monticola* DOUGL. F_1 hybrid displayed heterosis for growth traits, at age 11 while *P. cembra* x *P. wallichiana* JAKS. hybrid was intermediate between the 2 parents (BLADA, 1994);

– a recent diallel analysis (BLADA, 1995) within a natural population from the Carpathian, showed highly significant differences in growth traits for CGA, SCA, MAT and REC effects. The conclusion was that within the species *P. cembra* parents could be found with a good general combining ability, which could be used in a breeding programme;

This paper reports some results concerning genetic variation among – and within – 12 stone pine provenances at age 6.

[1]) A modified form of this paper will be presented at the XI World Forestry Congress, Antalya, Turkey, October 13 to 22, 1997.