Genetic Variability in Fourteen Provenances of Eucalyptus Species in Hawaii

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Summary

Eucalyptus spp. are among the most promising exotic tropical hardwoods under field testing for biomass crop production in Hawaii. A first step in establishing a genetically based tree improvement program aimed at increasing biomass yield, coppice regrowth, and other desirable traits for short rotation intensive culture of *Eucalyptus* spp. on potential biomass plantations is to determine the extent of genetic variability in the locally available stock of germplasm. This study presents an evaluation of allozyme variability revealed through gel electrophoresis in fourteen provenances involving five species of *Eucalyptus* introduced previously in Hawaii. The high levels of polymorphism and heterozygosity observed in our sample indicate that this initial stock of germplasm represents a suitable base for further genetic improvement. This information is critically important for developing and maintaining environmentally and economically sustainable biomass plantations for long-term success featuring stands which are not genetically vulnerable.

Key words: Eucalyptus spp. provenances, genetic variability, isozyme analysis, gel electrophoresis, biomass crops, Hawaii.

Introduction

Over 90% of the total energy use in Hawaii is supplied by imported oil, with more than 60% of this oil consumed as liquid fuels for transportation compared with only about 30% for electricity generation. The annual cost is over US$ 1 billion to HAWAII's economy. Therefore, the development of indigenous, renewable energy resources is a high priority in the state (PHILLIPS, 1990; PHILLIPS, CHUVELIOV and TAKAHASHI, 1992). By taking advantage of ideal conditions for plant growth, Hawaii could harvest sufficient fast-growing trees and grasses on less than 5% of its land area to provide all of the state's liquid fuel for ground transportation as methanol-from-biomass (PHILLIPS, NEILL and TAKAHASHI, 1988). Since the world oil-price shocks of the 1970's, researchers at the University of Hawaii's College of Tropical Agriculture and Human Resources and Hawaii Natural Energy Institute, the Nitrogen Fixing Tree Association, the Hawaiian Sugar Planters' Association, the Institute of Pacific Islands Forestry of the U.S. Department of Agriculture Forest Service, the BioEnergy Development Corporation, and others have initiated research and development projects to explore the feasibility of biomass crop production in Hawaii as a renewable and viable alternative to imported oil.

Two critical steps in the economic production of short rotation intensive culture (SRIC) biomass crops have been identified in Hawaii and elsewhere: (1) increasing biomass yield through genetically based tree improvement programs; and (2) reducing costs of biomass crop harvesting and handling through the development of appropriately scaled and designed SRIC harvesting systems (O'BRIEN and PHILLIPS, 1989). This study represents an initial step in addressing the former.

Because *Eucalyptus* spp. are widely adapted, fast-growing and high-yielding, they are planted extensively throughout the tropical and subtropical world to meet the increasing demand for paper pulp and fuelwood as a SRIC biomass crop (PENFOLD and WILLIS, 1961; HILLIS and BROWN, 1978). Over the past 2 decades, several provenances of *Eucalyptus* spp. including many species from different parts of Australia have been introduced in preliminary field trials on different islands of Hawaii to evaluate their adaptability and biomass yield potential (SCHUBERT *et al.*, 1988; DEBELL and WHITESELL, 1988). These bioenergy trials are in various stages of growth and evaluation. As a part of the evaluation program, 14 provenances belonging to 5 species were assessed for genetic variability using isozyme markers. Enzyme electrophoresis has been used as an aid to conventional provenance studies in many tree species (FALKENHAGEN, 1985; PEIRCE and BREWBAKER, 1973; YEH *et al.*, 1986).

The assessment of genetic variability is key to progress in tree improvement (ZOBEL, 1981). Information on levels of allozyme diversity can be very useful for determining on which species and provenances to begin genetic improvement through breeding programs (ADAMS, 1983). Because plantations consisting of genetically uniform material are highly vulnerable to major climating fluctuations or to epidemic insect and disease events, it is of paramount importance to evaluate and maintain genetic variability within the stands and breeding orchards for coping successfully with these crises and ensuring long-term stability. The base line information on allozyme variability among different provenances and species may be utilized for planning future, genetically-based tree improvement programs.

Materials and Methods

Fourteen provenances of five *Eucalyptus spp.* were included in this study. These species — *Eucalyptus grandis* (HILL) ex. MAIDEN, *E. saligna* SM., *E. camaldulensis* DEHNH., *E. urophylla* S. T. BLAKE, and *E. robusta* SM. — are in various field trials on the island of Hawaii in the State of Hawaii. These trials were established and are maintained by the Hawaiian Sugar Planters' Association and the BioEnergy Development Corporation (BDC), a wholly-owned subsidiary of C. Brewer and Co., Ltd., of Hawaii. The details on the origin, location, and number of trees sampled at the time of seed collected for these provenances are presented in *Table 1.*

Sample collection

Young leaves were collected in the field from a minimum of 30 trees per provenance. The leaf samples were numbered accordingly and preserved on ice in the field and transported the same day to the labo-

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Table 1. — Provenances of *Eucalyptus* spp. in Hawaii included in the study.

	Species	Provenance ID No.	Location where collected	Number of trees in collection
1.	*E. grandis*	MV 1	Local collection in Hawaii from earlier introductions	NA
2.	*E. grandis*	339-2	13 km north of Buladelah, NSW, Australia (32°20'S, 152°13'E)	10
3.	*E. grandis*	340-2	17 km north of Coffs Harbour, NSW, Australia (30°10'S, 153°8'E)	12
4.	*E. grandis*	341-2	Woondum, Queensland, Australia (26°17'S, 152°48'E)	8
5.	*E. grandis*	342-2	10 km south of Ravenshoe, Queensland, Australia (17°42'S, 145°36'E)	11
6.	*E. grandis*	343-2	24 km northeast of Atherton, Queensland, Australia (17°6S, 145°36'E)	22
7.	*E. saligna*	MV 2	Local collection in Hawaii from earlier introductions	NA
8.	*E. saligna*	344-1	South of Gympie, Queensland, Australia (26°25'S, 152°25'E)	20
9.	*E. saligna*	345-1	Consuelo T'Lands, Queensland, Australia (24°57'S, 148°3'E)	11
10.	*E. saligna*	346-1	Northwest of Kyogle, NSW, Australia (28°32'S, 152°46'E)	9
11.	*E. saligna*	347-1	Kroombit Tops, Queensland, Australia (24°25'S, 151°2'E)	25
12.	*E. urophylla*	MV 3	Gunung Negon, Island of Flores, Indonesia	NA
13.	*E. robusta*	MV 4	Local collection in Hawaii from earlier introductions	NA
14.	*E. camaldulensis*	278-6	Thailand	NA

Note: The past recording and present availability of definitive seedlot information on *Eucalyptus* spp. growing in Hawaii is limited.
NA = Not Available.

ratory at the University in Honolulu and stored at 4°C in a cold room for subsequent analysis within two days. A few provenances were sampled in the BDC field nursery where they were being hardened-off prior to outplanting.

Electrophoresis

Approximately 25 mg of fresh leaf tissue were homogenized in a few drops of chilled extraction buffer (Lebot et al., 1991) in wells carved in a Plexiplass block. The grinding was done quickly with a ground glass rod on ice The resulting slurry was absorbed onto Whatman #3 filter paper wicks (4 mm x 10 mm). The wicks were immediately loaded into 12% starch gels (Sigma Chemical Co., St. Louis, MO, USA) previously prepared in histidine-citrate buffer pH 6.5 (Cardy et al., 1983) and cooled to 4°C. Electrophoresis was conducted in a refrigerator at 4°C and 200 volts (20 v/cm) with 40 milliamperes for 6 hours. At the end of the electrophoresis, the gels were sliced horizontally into six laminae, stained, and assayed for six

Table 2. — Allele frequencies in different provenances of *Eucalyptus* spp. in Hawaii.
Notes: The sample size (n) = 30 for all provenances. Blanks indicate zero values. Species or proventnce numbers refer to *table 1*.

Locus & Allele		Species or Provenance													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pgi	A	0.117	0.067	0.050	0.133	0.017	0.383		0.017			0.017			0.183
	B	0.067						0.067				0.017			
	C	0.050	0.017	0.167		0.017	0.017	0.217	0.117	0.283	0.183	0.083		0.167	0.033
	D				0.067			0.017			0.033	0.200	0.083		0.133
	E													0.317	
	F	0.733	0.800	0.767	0.550	0.517	0.533	0.267	0.400	0.283	0.400	0.667	0.417	0.517	0.650
	G	0.033			0.183	0.067		0.267			0.200	0.017	0.150		
	H		0.117	0.017	0.067	0.383	0.067	0.100	0.250	0.033			0.233		
	I								0.217	0.083	0.033		0.117		
	J							0.067		0.317	0.150				
Pgm-1	A	0.150		0.033	0.150	0.067	0.183		0.067	0.017	0.117	0.233		0.333	
	B	0.300	0.133	0.300		0.017	0.167	0.267	0.017	0.200	0.283	0.433	0.250	0.067	
	C	0.550	0.867	0.667	0.717	0.867	0.650	0.733	0.917	0.783	0.600	0.333	0.667	0.517	0.600
	D				0.133	0.050							0.083	0.083	0.400

Locus & Allele		Species or Provenance													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pgm-2	A												0.383		
	B				0.033			0.033					0.167	0.033	0.300
	C	0.883	1.000	1.000	0.450	0.867	0.983	0.917	0.800	0.650	0.933	0.733	0.233	0.650	0.400
	D									0.050					
	E	0.117			0.367	0.133	0.017	0.050	0.183	0.300	0.067	0.267	0.217	0.233	0.217
	F				0.150				0.017					0.083	0.050
	G														0.033
6Pgd	A												0.050		
	B							0.250				0.050	0.200	0.167	
	C	0.917	0.767	0.850	0.917	0.900	1.000	0.333	0.983	0.883	0.683	0.717	0.733	0.683	0.617
	D										0.067	0.233	0.017	0.133	
	E	0.083						0.400	0.017		0.050			0.017	0.383
	F		0.233	0.100	0.017	0.100		0.017		0.067	0.183				
	G			0.050	0.067					0.050	0.017				

Locus & Allele		Species or Provenance													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Skdh	A				0.033				0.033		0.050	0.067		0.183	0.033
	B	0.017	0.033	0.017	0.133	0.050	0.050	0.238	0.233	0.033	0.03	0.250	0.050		0.083
	C	0.950	0.967	0.933	0.833	0.950	0.950	0.667	0.717	0.967	0.917	0.583	0.850	0.533	0.883
	D	0.033		0.050				0.050	0.017			0.100	0.100	0.100	
	E													0.033	
	F													0.150	
Idh	A		0.033	0.017			0.067					0.083	0.100	0.067	
	B	1.000	0.900	0.933	1.000		0.933	0.900	0.983	1.000	0.950	0.917	0.850	0.700	1.000
	C		0.050	0.050		1.000		0.100	0.017		0.050		0.050	0.133	
	D		0.017											0.100	
Mdh	A	0.283	0.417	0.233	0.317	0.117	0.100	0.417	0.650	0.550	0.433	0.217		0.033	
	B	0.333	0.583	0.767	0.683	0.883	0.900	0.583	0.350	0.450	0.567	0.783	1.000	0.967	0.883
	C	0.383													
	D														0.117

consistently interpretable enzyme systems following Shaw and Prasad (1970):
PGI (Phosphoglucoisomerase, EC 5.3.1.9);
PGM (Phosphoglucomutase, EC 2.7.5.1);
6 PGD (6-phosphogluconate dehydrogenase, EC 1.1.1.44);
SKDH (Shikimate dehydrogenase, EC 1.1.1.25);
IDH (Isocitrate dehydrogenase, EC 1.1.1.42); and
MDH (Malate dehydrogenase, EC 1.1.1.37).

Data analyses

Alleles were designated alphabetically in order of increasing mobility. Genotype frequencies were inferred

Table 3. — Genetic variability at seven loci in 14 provenances of *Eucalyptus* spp. in Hawaii.

Provenance[1] [ID No.]	Mean no. alleles per locus	Percentage polymorphic loci[2]	Mean heterozygosity: Direct count	Mean heterozygosity: HDYWBG expected[3]
1. *E. grandis* [MV 1]	2.7 (0.5)[4]	85.7	0.362 (0.135)[4]	0.311 (0.098)[4]
2. *E. grandis* [339-2]	2.4 (0.4)	71.4	0.210 (0.052)	0.242 (0.066)
3. *E. grandis* [340-2]	2.7 (0.4)	85.7	0.238 (0.065)	0.250 (0.064)
4. *E. grandis* [341-2]	3.0 (0.5)	85.7	0.281 (0.082)	0.377 (0.092)
5. *E. grandis* [342-2]	2.6 (0.5)	85.7	0.205 (0.066)	0.223 (0.070)
6. *E. grandis* [343-2]	2.3 (0.4)	71.4	0.214 (0.089)	0.220 (0.088)
Mean	**2.6**	**81.0**	**0.251**	**0.271**
7. *E. saligna* [MV 2]	3.3 (0.7)	100.0	0.357 (0.087)	0.457 (0.090)
8. *E. saligna* [344-1]	3.0 (0.4)	71.4	0.286 (0.090)	0.312 (0.097)
9. *E. saligna* [345-1]	2.7 (0.5)	71.4	0.362 (0.111)	0.339 (0.100)
10. *E. saligna* [346-1]	3.3 (0.6)	100.0	0.324 (0.081)	0.385 (0.097)
11. *E. saligna* [347-1]	3.1 (0.6)	100.0	0.333 (0.062)	0.443 (0.063)
Mean	**3.1**	**89.0**	**0.332**	**0.387**
12. *E. camaldulensis* [278-6]	3.3 (0.5)	85.7	0.370 (0.115)	0.411 (0.097)
13. *E. urophylla* [MV 3]	3.7 (0.4)	85.7	0.429 (0.077)	0.495 (0.076)
14. *E. robusta* [MV 4]	2.7 (0.5)	85.7	0.348 (0.093)	0.377 (0.092)
Overall Mean	**2.9**	**84.7**	**0.308**	**0.346**

[1]) The sample size (n) = 30 for all provenances.
[2]) A locus is considered polymorphic if the frequency of the most common allele does not exceed a value of 0.95.
[3]) Unbiased estimate based on HARDY-WEINBERG expected value (see NEI, 1978).
[4]) Standard errors are given in parentheses.

directly from the observed isozyme phenotypes. Seeds from controlled crosses or from selfing were not available for assay. Therefore, the genetic basis of the isozyme variation had to be inferred from the comparison of isozyme phenotypes observed in different provenances and species. Genetic studies of similar loci in other *Eu-*

calyptus species support the interpretation of the isozyme patterns in this study (MORAN and BELL, 1983). The observed allelic frequencies for the six enzyme systems were subjected to statistical analysis to compute various within-provenance variability measures such as mean number of alleles per locus, percentage of polymorphic loci, observed and expected levels of heterozygosity, and contingency chi-square analyses for homogeneity of gene frequencies between provenances (WORKMAN and NISWANDER, 1970). Unbiased genetic identity and distance coefficients (NEI, 1978) for all possible pair-wise combinations of provenances and species were computed to understand the genetic relationships between different species and provenances. A cluster analysis was performed on the genetic identity matrix with the unweighted pair group method using arithmetic averages (UPGMA) algorithm (SNEATH and SOKAL, 1973). All computations were performed using the computer program BIOSYS-1 (SWOFFORD and SELANDER, 1989).

The gene diversity analysis (NEI, 1973) was performed on the allele frequency data. The total gene diversity (H_T) was apportioned into gene diversity within provenances (H_S) and gene diversity between provenances (D_{ST}), where [$H_T = H_S + D_{ST}$]. H_T was calculated on the weighted average allele frequencies over all provenances. H_S is equal to the weighted average of the HARDY-WEINBERG expectation of heterozygosity over all provenances. D_{ST} is obtained by subtraction [$D_{ST} = H_T - H_S$]. The proportion of gene diversity between provenances is calculated as [$G_{ST} = D_{ST}/H_T$], where G_{ST} can vary between zero (when $H_S = H_T$) and one (when $H_S = 0$). Hierarchical gene diversity analysis was performed at the species level to understand the nature and distribution of genetic variability within and between different species in the study.

Results and Discussion

Allele frequencies and genetic variability

The observed allelic frequencies for the 6 enzyme systems encoded by seven loci across 14 provenances involving 5 species are presented in *table 2*. A total of 42 alleles were encountered and the number of alleles per locus varied from 4 in *Pgm-1*, *Idh*, and *MdH* to 10 in *Pgi*. Although there was a large number of low-frequency, species-specific alleles, all species shared the same major alleles. Contingency chi-square analyses (WORKMAN and NISWANDER, 1970) for homogeneity of gene frequencies between provenances demonstrated significant heterogeneity between provenances at all polymorphic loci ($P < 0.01$).

Within-provenance measures of genetic variability for different species are summarized in *table 3*. The mean number of alleles per locus varied from 2.3 to 3.0 with a mean of 2.6 for the species *E. grandis* while it was 2.7 to 3.3 with a mean of 3.1 for *E. saligna*. *Eucalyptus urophylla* recorded the highest mean for the number of alleles per locus (3.7). The species-wise mean percentage of polymorphic loci varied from 81.0% for *E. grandis* to 89.0% for *E. saligna* with *E. camaldulensis*, *E. urophylla*, and *E. robusta* being intermediate (85.7%). The mean observed heterozygosity value was highest for *E. urophylla* (0.429) followed by *E. camaldulensis* (0.370), *E. robusta* (0.348), *E. saligna* (0.332), and *E. grandis* (0.251). A similar trend was observed for the HARDY-WEINBERG expected levels of heterozygosity. The mean number of alleles per locus and heterozygosity levels observed for different provenances and species are quite high compared to many allogamous angiosperm trees including eucalypts (BROWN, 1979; HAMRICK et al., 1979; BURGESS and BELL, 1983; MORAN and HOPPER, 1983; SAMPSON et al., 1988).

Eucalyptus spp., being predominantly allogamous (PRYOR, 1976; MORAN and BROWN, 1980), recombine alleles from genetically diverse sources, including interspecific levels. The evidence for this comes from the occurrence of extensive hybrid swarms of *E. saligna* and *E. botryoides* on the south coast of New South Wales, Australia (PRYOR and JOHNSON, 1971), while occurrences of hybrid populations between *E. grandis* and *E. robusta* have been reported along the coastal areas of both New South Wales and Queensland, Australia (BURGESS and BELL, 1983). Natural hybrids between *E. grandis* and *E. saligna* have also been reported. The high levels of genetic variability in the

Table 4. — Matrix of unbiased genetic identity coefficients between species of *Eucalyptus*.

Species	Number of Provenances	Species				
		E. grandis	*E. saligna*	*E. camaldulensis*	*E. urophylla*	*E. robusta*
E. grandis	6	0.892 (0.725-0.986)[1]				
E. saligna	5	0.889 (0.708-0.976)	0.913 (0.864-0.967)			
E. camaldulensis	1	0.853 (0.752-0.921)	0.843 (0.813-0.876)	---[2] ---		
E. urophylla	1	0.876 (0.787-0.922)	0.860 (0.824-0.930)	0.888 (0.888-0.888)	--- ---	
E. robusta	1	0.861 (0.705-0.933)	0.845 (0.815-0.875)	0.914 (0.914-0.914)	0.883 (0.883-0.883)	--- ---

[1]) Standard errors are given in parentheses.
[2]) Dashed line (---) indicates no comparisons.

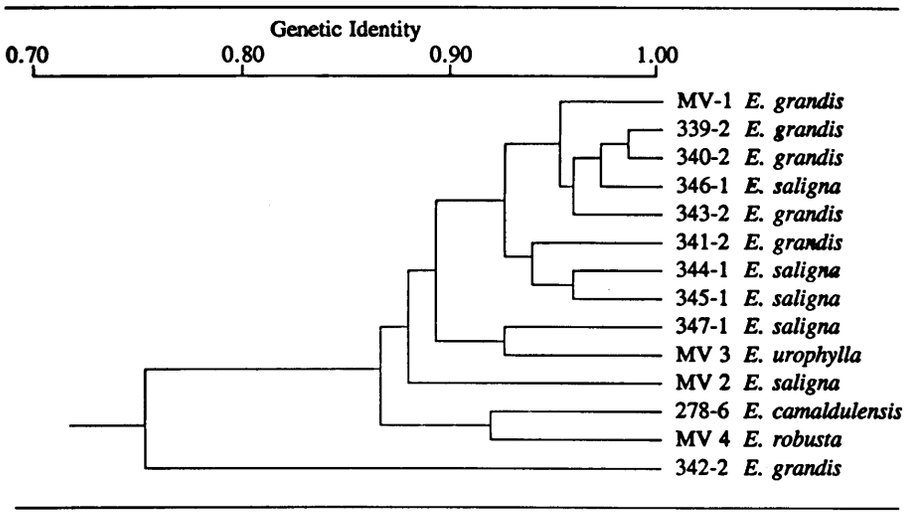

Figure 1. — Dendrogram indicating the genetic relationships among different *Eucalyptus* species and provenances in Hawaii.

present study may reflect exchange of genes on levels up to and including gene flow among species.

Genetic relationship between species

The genetic relationships between the species and provenances were quantified by computing the unbiased genetic identity and distance statistics proposed by Nei (1978). Because of the large size of the data set, only unbiased genetic identity and distances for all possible pair-wise species comparisons is presented (*Table 4*). The *E. grandis* provenance (ID No. 342-2) originating from Ravenshoe, Queensland, Australia, exhibited the lowest average unbiased genetic identity (0.752), followed by the *E. camaldulensis* provenance (ID No. 278-6) from Thailand with 0.871, and the *E. robusta* provenance (ID No. MV 4) with 0.861. The average genetic identity values observed within and between *Eucalyptus* spp. in the present study indicate that although the provenances harbor high levels of within-provenance variability, they share a large proportion of the alleles enumerated for different loci. The average species pair-wise genetic identity coefficients ranged from 0.843 between *E. camaldulensis* and *E. saligna* to 0.914 between *E. robusta* and *E. camaldulensis*.

The genetic identity values in the present study are in the range expected for conspecific populations of allogamous species (Hamrick et al., 1979; Dancik and Yeh, 1983). The *E. grandis* provenance (ID No. 342-2) from Ravenshoe, Queensland, Australia, has formed a separate cluster in the UPGMA cluster analysis at approximately the 75% level of similarity indicating that it is relatively highly divergent from all other provenances and species in the study (*Figure 1*). Although the rest of the provenances exhibit high within-provenance variability, they appear similar to each other. *Eucalyptus grandis* and *E. saligna*, which both belong to the subgenus *Symphyomyrtus*, section *Transversaria*, series *Salignae*, and subseries *Saligninae* (Pryor and Johnson, 1971), exhibited a close genetic relationship.

Partitioning of genetic variability

The total genetic variability (H_T) was partitioned into variability within provenances (H_S) and between prove-

Table 5. — Measures of gene diversity and partitioning of genetic variability.

Measures of gene diversity					Partitioning of genetic variability		
Locus	H_T	H_S	D_{ST}	G_{ST}	Within provenances	Between provenances within species	Between species
Pgi	0.676	0.588	0.088	0.130	0.870	0.074	0.057
Pgm-1	0.500	0.439	0.061	0.123	0.877	0.076	0.047
Pgm-2	0.411	0.324	0.087	0.212	0.789	0.079	0.133
6Pgd	0.374	0.309	0.064	0.174	0.826	0.107	0.067
Skdh	0.291	0.257	0.035	0.119	0.881	0.050	0.069
Idh	0.246	0.117	0.128	0.523	0.477	0.463	0.060
Mdh	0.444	0.347	0.096	0.217	0.783	0.102	0.115
Mean	0.420	0.340	0.080	0.214	0.809	0.114	0.077

Note: H_T = total gene diversity; H_S = gene diversity within provenances; D_{ST} = gene diversity between provenances; G_{ST} = proportion of total gene diversity due to genetic differences between provenances.

nances (D_{ST}) to understand the distribution of genetic variability (*Table 5*). The mean total genetic diversity (H_T = 0.420) and the mean proportion of the diversity between provenances (G_{ST} = 21.4%) in the present study are higher than that previously reported for widespread eucalypt species (0.190 to 0.272) (MORAN and HOPPER, 1987). Although 5 species of *Eucalyptus* were included in the provenance analysis, on the average nearly 80% of the total gene diversity was found within provenances. The average level of within-provenance variability did not change when the total variability was apportioned into variability within provenances, between provenances within species, and between species. The proportion of total variability due to genetic differences among species accounted for only about 8%. The high levels of heterozygosity suggest that the provenances would respond to selection.

Conclusion

Our study of allozyme diversity identified high levels of polymorphism and heterozygosity in the introduced *Eucalyptus* spp. and provenances under field testing in Hawaii, as compared to other allogamous plant groups, with a large proportion (80%) of total variability found within provenances. Therefore, this initial stock of germplasm represents a promising base for further tree improvement through selection. A long-range breeding program should not overlook, however, further introductions of other sources of *Eucalyptus* germplasm.

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