Discriminant Analysis of Eucalyptus cinerea x Eucalyptus maculosa Hybrids[1]

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Introduction

The importance of hybrid trees in afforestation programs is well documented, and considerable effort has been devoted to the production of artificial hybrids. In addition, studies of natural and artificial hybrids yield information on the phylogenetic relationships of forest trees, although exact taxonomic analysis of some hybrid progenies is difficult.

The value of multiple measurements in taxonomic problems was early recognized by Fisher (1936) and, in describing the morphology of hybrid trees, it is customary to use several variables to distinguish the hybrids from their parents. When little information is available on the variation pattern or the degree of genetic control of individual characters, it is desirable to use a combination of several diagnostic characters.

The development of electronic computers has facilitated the use of different types of statistical multivariate analysis for biological problems, and a valuable technique for such studies is discriminant analysis (Tomassone, 1963), although it has rarely been employed with forest trees. Two forms of locust (Robinia *pseudoacacia* L.) were separated by a discriminant function (Hopp, 1941) and Clifford and Binet (1951) developed discrimination theory to calculate formulae for taxonomic classification of the members of a hybrid swarm between *Eucalyptus elaeophora* and Eucalyptus goniocalyx. Principal component analysis was used by Gardiner and Jeffers (1962) to analyze a number of groups of the collective species *Betula* alba, and Hatheway (1962) computed a weighted hybrid index for a canonical analysis of violets.

Mergen and Furnival (1960) used a stepwise discriminant analysis of 27 characters for hybrid seedlings of Pinus *thunbergii* X Pinus *densiflora*. This enabled the isolation of groups of variables that discriminated best between the parents, and that determined traits in the hybrid that were either greater than the parental average or intermediate between the parents. In this type of analysis, the overall behavior of a large number of characters was considered, rather than the absolute magnitude of a few unrelated traits, and the authors considered it likely to be of great value in forest biology research. This would be particularly true in the early stages of a species hybridization program, where it is desirable to obtain information on the overall appearance and development of the hybrid plants.

To verify the usefulness of the technique, and to expand its application to include broadleaved species, a similar analysis was performed on hybrids in the refractory genus Eucalyptus (Myrtaceae); a total of 27 morphological and anatomical traits were used for analysis of seedlings of *Eucalyptus cinerea* F. v. M. X *Eucalyptus maculosa* R. T. Baker. In addition, observations were made of the variation in certain physiological and cytological characters, but these were not included in the discriminant analysis.

Literature review

Taxonomic relationships among Eucalypts are complex, and some 603 species, subspecies, varieties and hybrids have been recognized (see Blakely, 1955; Hamilton, 1961). Difficulties in classification have been caused, in part, by the absence of features that are uniform within a species and distinct between species. Also, the occurrence of hybrid and clinal forms has generally been overlooked, and these types have often been classified as species.

The flowers of Eucalypts are protandrous, thus favoring outcrossing and the possibility of interspecific hybridization. Controlled pollination experiments have shown that hybridization can occur (e. g. Pryor, 1951 a), and the evidence of segregation in open-pollinated progeny tests suggest that hybridization does occur naturally, with characteristics of the F, hybrids being generally intermediate between the parental types (Hamilton, 1961). According to Pryor (1951 b), many of the types of some "species" are members of hybrid swarms, and other workers have analyzed hybrid swarms between several pairs of species (e. g. Clifford, 1954; 1955; 1960; Clifford and Binet, 1954; Pryor, Chattaway and Kloot, 1956; Pederick, 1961).

Both of the parent species used in the present research have been found in natural hybrid swarms. Among hybrids of Eucalyptus *elaeophora* X Eucalyptus macubosa, individuals were either intermediate between the parent species or similar to one parent (Pryor, Chattaway and Kloot, 1958). Similar observations were made on hybrids between *Eucalyptus viminalis* and Eucalyptus *cinerea* (Pryor, 1951 c). In addition Pryor (1954, 1956) has recorded the occurrence of intermediate hybrids between *Eucalyptus* cinerea and both *Eucalyptus* blakelyi and Eucalyptus *robusta*. Progeny of a hybrid Eucalyptus *rubida* X Eucalyptus maculosa closely resembled Eucalyptus *maculosa* in resistance to a leaf eating beetle (Pryor, 1952).

The two species Eucalyptus *cinerea* and Eucalyptus *maculosa* do not hybridize naturally because the former flowers in the spring, the latter in the autumn. However, an F, hybrid has been produced after storing pollen at -16^0 C for the six months interval (Boden, 1958).

Materials and methods

Eucalyptus cinerea and *Eucalyptus* maculosa are native to the eastern parts of Victoria and New South Wales in southeast Australia; their natural ranges overlap at higher elevations (1,000—4,000 ft.) in the Australian Alps. Both occur as trees from 20 to 60 ft. high but they are of poor timber quality; they are used in Australia and in the Mediterranean region mainly for fencing and firewood.

The seeds used in the present study were obtained from open- and controlled cross-pollinations of one tree of both species, carried out by Professor Lindsay Pryor at Canberra University College. The seeds were sown during December, 1959 in a greenhouse at New Haven, Connecticut, U. S. A., and observations were made immediately after germination and again in December, 1960, on eight plants of open-

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pollinated *Eucalyptus cinerea,* eight of *Eucalyptus maculosa* and 13 hybrid individuals. The following greenhouse and laboratory measurements were made:

Cotyledon characteristics: — One cotyledon was selected randomly from each seedling one month after seed germination. All measurements were made to the nearest 0.1 mm with a binocular stereoscopic microscope at a magnification of 10×. The *length* was measured at the longest point, and the *width* was recorded at the junction of the cotyledon and petiole. The *ratio* of length to width was calculated. The *wing tangent* was measured as the distance between the outer edge of the cotyledon at a point opposite the junction of petiole and cotyledon, and a line tangent to the outer edge of the wing.

Leaf characteristics: — The ten most distal leaves were selected from the longest branch of the whorl nearest the middle of the tree. *Leaf arrangement* was classified as alternate, opposite or intermediate. The *length* of each leaf was measured to the nearest millimeter along the central vein, and the *width* was determined at the widest point; the *ratio* of length to width was calculated. The *apex angle* and *base angle* were obtained to the nearest degree at the intersections of the central vein and leaf edge. The *number of veins* was counted under a binocular microscope fitted with a square grid measuring 8.75 mm at 7×; veins were included if they occurred within the grid and averaged more than one division (1.55 mm) from the central vein. The same grid was used at 25× to determine the number of oil glands on the upper surface near the middle of the leaf. To determine the *number of stomates,* small pieces of leaf (approximately 3 sq mm) were cut from the center of each leaf, boiled in Chlorox, and stored at 60° C for 3—4 hours until the chlorophyll disappeared. After washing in water, the epidermal layer separated; this was stained in 1% safranin for 30—60 seconds and mounted on glass slides in Karo syrup. The number of stomates was counted in three microscope fields at 450×. *Stomatal length and width* were measured with a filar micrometer for five stomates on each of five slides per tree and the *ratio* of length to width was calculated.

Stem characteristics: — The *total height* of each seedling was measured in centimeters from the top of the pot. *Stem diameter* was recorded in millimeters at 5 cm above the soil, and at ¼, ½, ¾ of the total height of the tree. The *total number of nodes* and *number of branches* were recorded on each tree. *Stem color* was determined at the midpoint of the stem in bright sunlight, using Munsell color charts. Data on *oil glands* were secured from a portion of bark (approximately 2 × 5 mm) taken from the middle of the stem. The *number of glands* was counted under a binocular microscope at 25× and converted to *number per unit area. Gland height* and diameter were measured with a filar micrometer at 125×. For the *ligno tuber,* width was determined to the nearest centimeter at the widest point (A) and at the mid point (B), and *height* was obtained at the longest point (C). *Tuber longitudinal area* was derived from the formula:

$$\text{Area} = \frac{(A + B}{2} \times 0.5C) + (B \times 0.5C)$$

Cross-sections of $60-90\,\mu$ thickness were cut from the stem of every tree. One section per tree was stained in safranin and mounted in balsam. The *number of vessels* was counted in four squares of the ocular grid at 25×. The *diameters* of 20 vessels occurring approximately along one radius were measured to the nearest micron at 845×. The *length* and *width of the pith* were determined by filar mi-

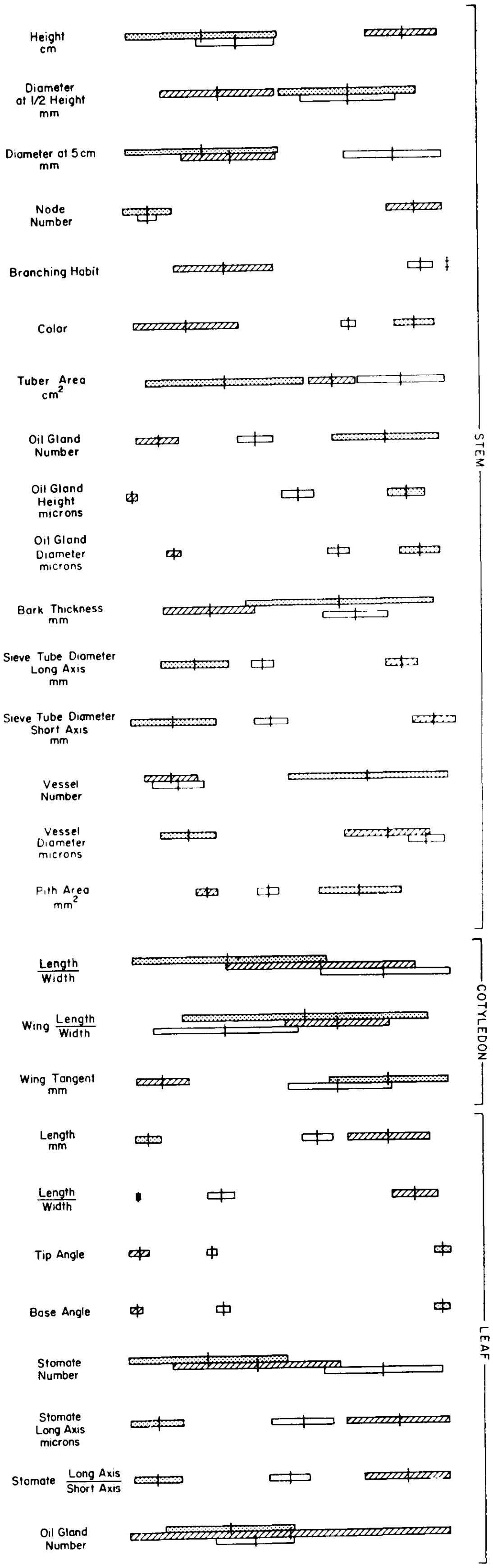

Figure 1. — Mean values (± one standard error) for 27 characteristics in parental and hybrid progeny groups.
Stippled = *Eucalyptus cinerea*
Open = *Eucalyptus cinerea* × *Eucalyptus maculosa*
Cross-hatched = *Eucalyptus maculosa*

crometer to the nearest 0.1 mm at 66×. *Bark thickness* was recorded from four measurements per section, also at 66×. *Sieve tube width* was measured in the shortest and longest directions on each of five sieve tubes in the third or fourth row of tanniferous parenchyma.

The data were anayzed by an IBM 650 electronic computer to determine the traits:

1) that discriminated best between the two parental species,
2) with values larger for the hybrid than the average of the parental species,
3) with values for the hybrid intermediate between the parental species.

The method previously described by MERGEN and FURNIVAL (1960) was used for this analysis, and 27 characteristics were employed in a stepwise process for each of three dummy variables.

Results and Discussion

Discriminant Analysis

The values (mean ± one standard error) for the 27 characteristics in the parental and hybrid groups are presented in *Figure 1.* The ranges of values for hybrids generally overlapped those of one parent in characters associated with overall appearance, such as stem height, node number, and stem diameter at half height. However, in stem diameter at 5 cm, and in area of the ligno-tuber *(Figure 2)*, the hybrids were superior to both parents, while in branching habit and stem color the three progeny groups were clearly distinct with the hybrids intermediate. The three groups were separated in the majority of other stem characters but there was considerable overlap between the ranges of *Eucalyptus maculosa* and the hybrids in vessel characters, while the hybrids resembled *Eucalyptus cinerea* in bark thickness.

Cotyledon characters showed no systematic pattern of variation, but the hybrids were commonly intermediate in leaf traits. Only in the number of stomates was the hybrid value greater than either parental mean. In the number of oil glands, the means were similar for the three groups, although the hybrids were less variable than the parents. The intermediacy of the hybrids in several stem and leaf traits is illustrated in *Figure 3.* It is of particular interest to note the intermediacy in phyllotactic arrangement; in *Eucalyptus cinerea* leaf arrangement was opposite, in *Eucalyptus maculosa* it was alternate. Both types of phyllotaxy appeared on the same hybrid individual.

The results of the stepwise screening process of the three discriminant regression analyses are shown in *Table 1.* The entries are the percentages of variation in the dummy variables accounted for by each measured variable after adjustment for the main (italicized) variables heading the group above it.

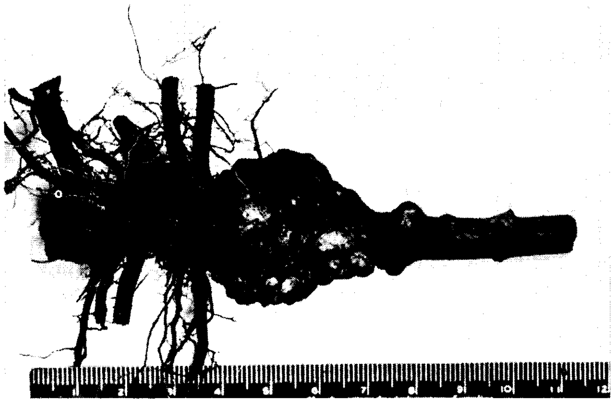

Figure 2. — Ligno-tuber in *Eucalyptus cinerea.*

Thus, stem color accounts for 50.0% of the variation in Y_3 (hybrid intermediate) when considered alone, for 3.1% after adjustment for leaf base angle, and for 0.5% after adjustment for leaf base angle, stomate ratio, and leaf tip angle. A total of 99.1% is accounted for by these four variables together with the diameter of stem oil glands and the leaf ratio, but clearly leaf base angle is the major component, accounting for 88.7% of the total variation.

The same character allowed virtually complete (98.6%) separation of the parental species (Y_1). Leaf tip angle accounted for an additional 0.7% but when used alone it contributed 97.4% of the total variation. Three other characters when used alone, facilitated accurate distinction of the parents — oil gland height (93.3%), leaf ratio (91.6%) and oil gland diameter (90.6%).

A total of seven characters accounted for 92.3% of the variation in the dummy variable associated with hybrid vigor (Y_2), although a maximum of only 28.2% was attributed to any one character alone. Node number contributed 34.8% after adjustment for vessel diameter.

Many of the discriminators for hybrid vigor may be considered to reflect the general vigor of the trees, e. g. node number and stem diameter. This form of hybrid vigor is perhaps surprising because the hybrid seeds were smaller than those of either parent. Fifty hybrid seeds weighed 28.9 mg, compared with 36.4 and 32.6 mg for *Eucalyptus cinerea* and *Eucalyptus maculosa,* respectively. However, it should be noted that hybrid vigor as used here was the superiority of the hybrid value over the mean parental value. Many of the discriminators of hybrid intermediacy were morphological or anatomical traits, e. g. leaf angles, stomate and leaf ratios, or oil gland diameter.

Essential oils

The inheritance of biochemical traits in forest trees has recently received considerable attention and, in F_1 hybrids of pines, turpentine composition was shown to be either intermediate between the parental types (BANNISTER, BREWERTON and MCDONALD, 1959; FORDE, 1964) or more like one parent (MIROV, 1956). Variations in yield and physical properties of essential oils in the leaves of *Eucalyptus* are important taxonomic traits that are strongly inherited and capable of recombination in hybrids (PRYOR and BRYANT, 1958).

To investigate the essential oils in *Eucalyptus cinerea, Eucalyptus maculosa* and their hybrids, composite leaf samples from four or five plants were used. Two samples were available from both parental species and three samples from the hybrid.

Steam distillation is regarded as the most reliable method for extracting essential oils (GUENTHER, 1948) and this method was used. Charges for distillation were prepared by cutting the leaves into pieces approximately 0.5 sq. in. The pieces for each sample were placed on a two-inch layer of glass wool in separate flasks. The charges ranged in weight from 85 to 132 grams.

Steam was passed through the glass wool and the leaf charge to vaporize the essential oils. The vapors were passed through a cold water condenser and the condensate was collected in a 100 cc buret. The buret was adjusted so that the stream of water released through the stopcock balanced in the inflow of condensate. The pale yellow, immiscible

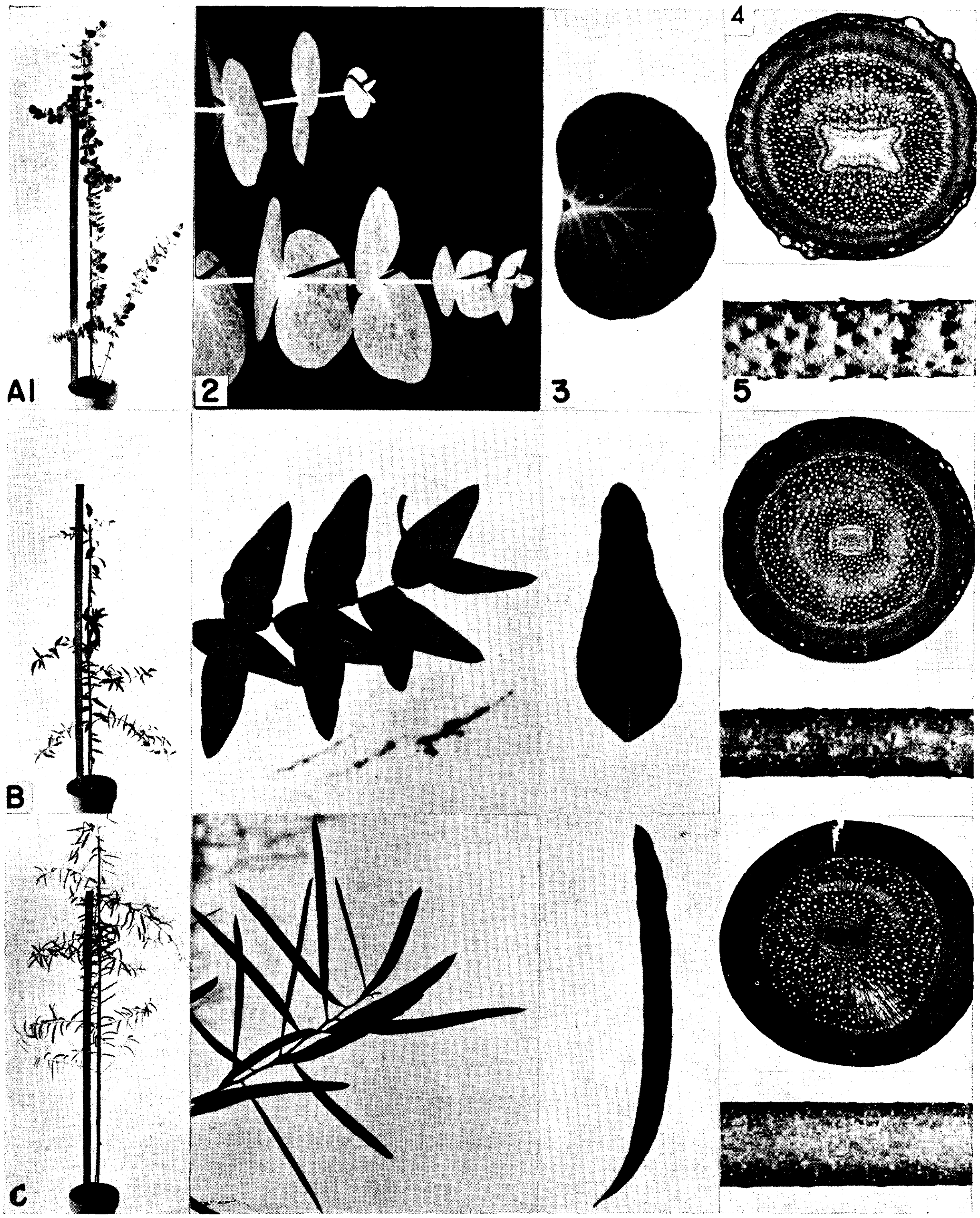

Figure 3. — Comparison of parents and hybrid. — A1-5. *Eucalyptus cinerea:* 1. Seedling; 2. Leaf arrangement; 3. Leaf; 4. Stem cross-section; 5. Stem with oil glands. B1-5. *Eucalyptus cinerea* × *Eucalyptus maculosa.* C1-5. *Eucalyptus maculosa.*

oils collected on the surface of the reservoir of water maintained in the buret.

Distillation temperatures varied from 97.5° C at the start of distillation to 99.5° C at the finish. Distillation of the first sample was continued until oil droplets in the condensate were minute and infrequent. Subsequent distillations were carried on for periods in proportion to the weight of the charge. In all cases, oil droplets in the fresh condensate were minute and infrequent at the end of the distillation period.

Oil yield was expressed in ml per 100 g fresh weight of leaf. The mean value for the hybrid (0.65) was similar to that of *Eucalyptus maculosa* (0.67), and approximately half that of *Eucalyptus cinerea* (1.26). However, the numbers of leaf oil glands were similar in the three groups.

Refractive indices were determined with an Abbe refractometer at 25° C. A correction factor of 0.00044 per degree Centigrade was used to compare the oils at standard temperature (20° C; LANGENAU, 1948). The refractive index for

Table 1. — Stepwise Discriminant Analysis of *Eucalyptus cinerea* × *Eucalyptus maculosa*

Y_1: Difference between parents		
Leaf base angle	98.6%	
Leaf tip angle		97.4%
Stem oil gland height		93.3
Leaf Ratio Length/Width		91.6
Stem oil gland diameter		90.6
Node number		79.2
Leaf length		69.4
Sieve tube width-long axis		68.1
Sieve tube width-short axis		68.0
Stomate ratio Length/Width		66.6
Branching habit		59.7
Stomate length		55.1
Stem color		53.1
Vessel diameter		52.4
Stem oil glands per unit area		50.5
Pith area		49.1
Wing tangent		47.4
Vessel number		29.6
Total plant height		29.3
Stem diameter at ½ height		12.7
Leaf tip angle	0.7%	
Pith area		0.4%
Wing tangent		0.4
Leaf oil gland number		0.3
Sieve tube width-long axis		0.3
Node number	0.3%	
	99.6%	
Y^2: Hybrid superior to mid-parent value		
Vessel diameter	28.2%	
Node number		26.2%
Stem diameter at 5.0 cm.		20.5
Tuber volume		12.8
Node number	34.8%	
Bark thickness		25.2
Leaf ratio Length/Width		24.1
Stem oil gland height		22.1
Stem oil gland diameter		19.4
Sieve tube width-long axis		14.8
Sieve tube width-short axis		14.0
Total plant height		11.2
Stomate ratio Length/Width	11.6%	
Leaf base angle		11.3
Leaf tip angle		10.3
Stem diameter at ½ height		7.5
Stem diameter at ½ height	4.5%	
Bark thickness		3.9
Stem color		3.7
Total plant height		3.1
Stem diameter at 5.0 cm.	5.5%	
Bark thickness		4.1%
Stem color		3.6
Bark thickness	5.2%	
Stem color		2.9
Leaf tip angle	2.5%	
	92.3%	
Y_3: Hybrid intermediate between parents		
Leaf base angle	88.7%	
Leaf tip angle		86.8%
Leaf ratio Length/Width		85,2
Stem oil gland diameter		82.4
Stem oil gland height		81.1
Sieve tube width-long axis		60.8
Sieve tube width-short axis		60.4
Leaf length		59.9
Stomate ratio Length/Width		57.0
Node number		56.3
Stem color		50.0
Branching habit		48.9
Pith area		45.0
Stem oil glands per unit area		44.5
Stomate length		40.1
Vessel diameter		32.4
Wing tangent		23.7
Vessel number		23.2
Total plant height		19.6
Stomate ratio Length/Width	7.2%	
Leaf ratio Length/Width		7.2%
Stem oil gland diameter		5.9
Stem oil gland height		4.0
Branching habit		4.0
Stem color		3.1
Wing tangent		2.6
Tuber longitudinal area		2.3
Sieve tube width-long axis		1.9
Sieve tube width-short axis		1.9
Leaf tip angle	1.7%	
Leaf length		1.3%
Stem oil gland diameter		1.0
Bark thickness		0.8
Stem oil gland diameter	0.9%	
Stem color		0.5
Leaf ratio Length/Width		0.5
Stem oil gland height		0.5
Stem color	0.3%	
Leaf ratio Length/Width		0.3
Leaf ratio	0.3%	
	99.1%	

the hybrid (1.4642) was greater than that for either parent (*Eucalyptus cinerea*, 1.4604; *Eucalyptus masculosa*, 1.4624). The parental values are both slightly lower than those (1.4649 and 1.4684) obtained by PENFOLD and MORRISON (1950).

The optical rotation of each sample was determined in a standard Lippich half-shadow polarimeter, fitted with a 200 mm tube. A dilution of 1 : 100 essential oil in xylene was necessitated by the small amounts of oil distilled; any experimental error in reading the rotation was thus magnified 100 times. This, together with the small number of samples used, may acount for the comparatively large values obtained with this material. According to PENFOLD and MORRISON (1950), the optical rotations for *Eucalyptus cinerea* and *Eucalyptus maculosa* are + 2.5 to 4.1° and + 2.3 to 3.4°. Here the rotations were 9.6° and 10.6°, with a value of 7.5° for the hybrid. It is possible that this discrepancy is the result of environmental modification, or that it occurs because leaves of different developmental stages were used by the different investigators.

Chlorophylls

Acetone extracts of chlorophylls from two macerated 5-gm samples in each progeny group were analyzed by spectrophotometer (MACKINNEY, 1941). In average total chlorophyll content, the hybrids (4.35 mg/g dry weight of leaf) were intermediate between *Eucalyptus cinerea* (4.95 mg/g) and *Eucalyptus maculosa* (4.15 mg/g). However, the proportion of chlorophyll B was higher in the hybrids (ratio A : B = 1.719, compared with 2.077 and 2.633 in the parental species).

Photosynthesis and respiration

Three branch samples, each bearing 1—2 g of leaves, were obtained from each of the three progeny groups. These were introduced in the lucite chamber of a Liston-Becker infrared gas analyzer (see Bourdeau and Mergen, 1959, for experimental details), and a stream of air containing 300 ppm carbon dioxide (CO_2) was passed through the chamber at a rate of 3 liters per minute. The rate of uptake or loss of CO_2 in micrograms per minute was determined at each of five light intensities: 0 (i. e. dark), 175, 500, 3500 and 7600 ft-c.

The mean rate of CO_2 transfer was expressed in terms of fresh weight, dry weight, and area of the leaves, but in all cases the relative reactions to changing light intensity were the same for the three progeny groups (e. g. dry weight, *Figure 4*). In darkness the samples from hybrid seedlings respired at a greater rate (38 μg/min/g dry weight) than either parent (20 μg). At light intensities of 175 and 500 ft-c, photosynthesis occurred at a slightly greater rate in the hybrids than in the parents. At an intensity of 3,500 ft-c the hybrids were clearly superior to the parents, and the photosynthetic rate of *Eucalyptus maculosa* was significantly greater than that for *Eucalyptus cinerea*. This intensity appeared to be the optimum for *Eucalyptus cinerea* and the hybrid material; the photosynthetic rates for these progeny groups decreased markedly at an intensity of 7,000 ft-c. Only leaves of *Eucalyptus maculosa* showed an increased rate, and when expressed on the basis of leaf area, the rate for this species also decreased.

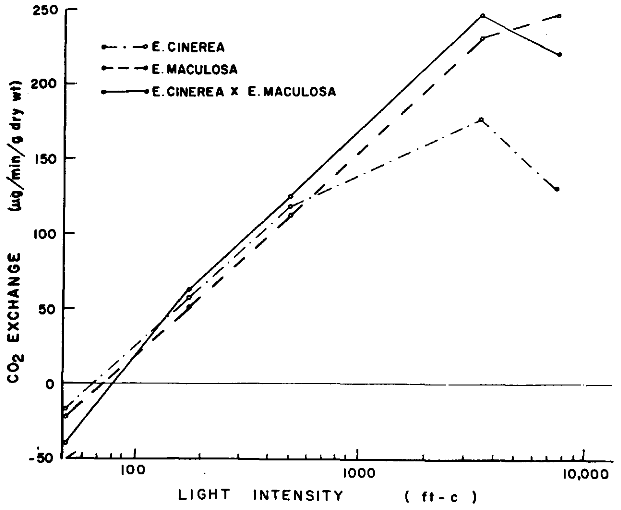

Figure 4. — Relationship of photosynthetic and respiratory rates to light intensity.

The superiority of the hybrid material in photosynthesis corroborates the evidence for some degree of hybrid vigor in certain morphological traits.

Chromosomes

The chromosomes in *Eucalyptus* are small, and karyotypes have not been analyzed in detail. However, the diploid number has been determined as 2n = 22 for many species (see Atchinson, 1947; Ruggeri, 1961 b), including *Eucalyptus cinerea* (Ruggeri, 1960, 1961 a and b); in several species the diploid number has been reported as 2n = 24.

To determine the diploid chromosome number of *Eucalyptus cinerea, Eucalyptus maculosa* and their hybrid, smears were made of meristematic tissues in young axillary buds. The buds were fixed in 1 : 3 acetic-alcohol and hydrolyzed for 15 minutes in normal hydrochloric acid. The tissues were smeared in aceto-orcein and metaphase plates were photographed and drawn by camera lucida *(Figure 5)*. The small size of the chromosomes precluded karyotypic analysis but in both parents and hybrids, the diploid number was 22.

No gross morphological differences were detectable between the karyotypes and it is probably that individual and specific variation has occurred at the single gene level.

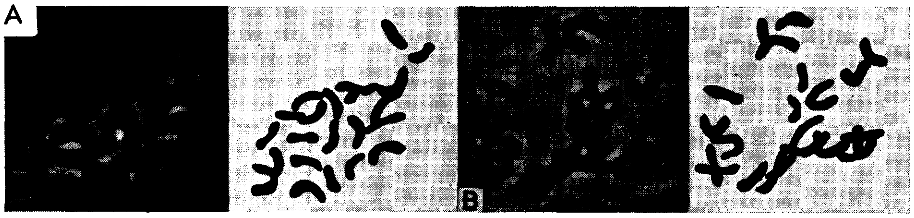

Figure 5. — Photomicrograph (left) and camera lucida drawing (right) of chromosome complement in : A. *Eucalyptus maculosa* (1940×); *B. Eucalyptus cinerea* (2125×).

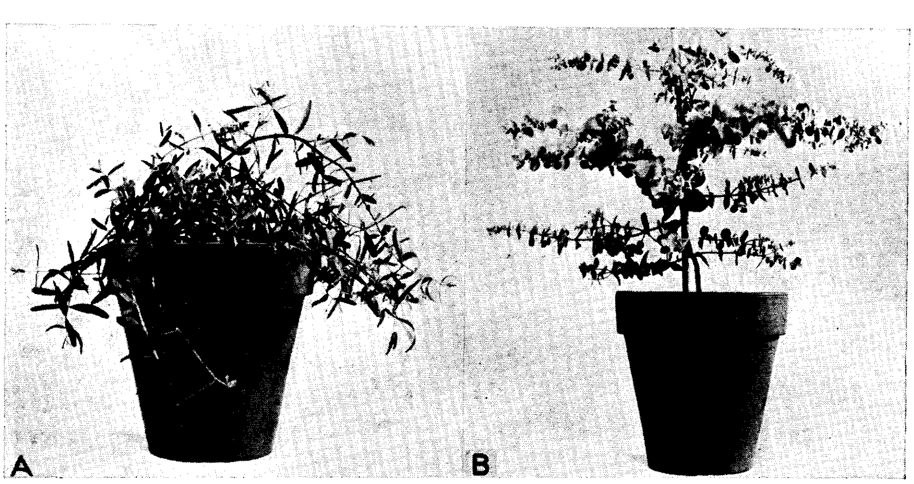

Figure 6. — Mutant seedlings (in 9" pots). A. Procumbent form of *Eucalyptus cinerea* × *Eucalyptus maculosa*. B. Dwarf form of *Eucalyptus cinereu.*

Occasional mutant forms were observed among spare seedlings and two are illustrated in *Figure 6*, — a procumbent form of the hybrid (A) and a dwarfed, densely branched form of *Eucalyptus cinerea* (B).

Conclusions

Hybrid seedlings of *Eucalyptus cinerea* × *Eucalyptus maculosa* were intermediate between the parents in 19 of the 27 characters studied. The angles of the leaf tip and of the leaf base were reliable indices for separating both parents and hybrids. These characters were easily determined, and relatively little precision was added by including additional variables in either of the regressions Y_1 or Y_3. In Y_2, however, no individual character showed a high degree of discrimination and at least six variables are necessary to account for 90% of the variation. Further, in the regression Y_2, the hybrids were superior to the mean of the parental values, not to the maximum parental value: in the remaining 8 of the 27 characters, the hybrids were identical with one parent in the two traits (node number and diameter at half height) that would be considered typical indices of hybrid vigor. This emphasized the intermediacy of the hybrid.

The results obtained with one-year seedlings do not necessarily correspond to those derived from mature trees; this is praticularly the case with leaves in the genus *Eucalyptus*, where several distinct developmental stages are found. However, this type of discriminant analysis appears suitable for comparisons of the overall reaction of a large number of characters, and it facilitates the determination of individual traits or small numbers of traits that isolate the two parental types and their hybrids.

In the present study, only one variable (leaf base angle) was required to account for nearly 90% of the variation in the regression for hybrid intermediacy. By contrast, a total of five characters was required to account for the same percentage of variation in a similar study using pines (Mergen and Furnival, 1960). Thus, the value of a discriminant analysis varies with the species involved.

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