Open-pollinated, interspecific F1 hybrid seed orchards of *Leucaena*
II. *Analysis of seedlings*

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Abstract. Seed produced on a single clone of a self-incompatible *Leucaena* species interplanted with cross compatible pollen donors of another species should theoretically be interspecific hybrid seed. This paper reports on the parentage of seed produced on two clones (K804, K953) of *L. pallida* produced in two interspecific seed orchards during the first year of growth. Grafting was used to propagate the two *L. pallida* clones and the interplanted pollen donors including *L. leucocephala* (K420, K481, K636) and the F1 hybrid KX3 (*L. diversifolia* K156 × *L. leucocephala* K636). Seed was produced on *L. pallida* K804 in one orchard, and on *L. pallida* K953 in the other orchard. Seeding leaf morphological characteristics including pinnule pairs per pinna, pinnule width and general appearance were used to determine parentage of seed produced on the *L. pallida* clones in each orchard. *L. pallida* K804 produced 81% interspecific hybrid seed while *L. pallida* K953 only produced 27% hybrid seed. These results suggest a breakdown of the gametophytic self-incompatibility mechanism in *L. pallida*, particularly in the K953 clone. The feasibility and possible improvement to this seed production system are discussed.

Introduction

Species of *Leucaena* play a prominent role in agroforestry systems throughout the tropics (Brennan et al., 1998). Prior to the 1980s there were no reports of major pest or disease problems within the genus. However, in 1984 the Leucaena psyllid (*Heteropsylla cubana*) spread throughout much of the tropics and caused severe defoliation and losses, especially to susceptible lines of *L. leucocephala* (Wheeler et al., 1994). As a result, breeders began to evaluate other *Leucaena* spp. and interspecific hybrids for psyllid resistance. A primary breeding objective has been to incorporate the exceptional fodder qualities and fast growth of *L. leucocephala* with the psyllid resistance found in several other species (*L. pallida*, *L. diversifolia*) (Brewbaker and Sorensson, 1990). Several interspecific hybrids produced by hand-pollination, outperformed both parental species and showed promise as alternatives to cultivars susceptible to the psyllid (Austin et al., 1997; Sorensson et al., 1994). One of the most promising self-compatible (SC) hybrids was *L. pallida* × *L. leucocephala* which has rapid growth (Sorensson et al., 1994), high quality fodder (Wheeler et al., 1994) and psyllid resistance (Austin et al., 1997).

Bray (1984) proposed a cost effective, large-scale scheme to produce inter-specific F1 hybrid seed through open pollination. In this scheme, an individual of a self-incompatible (SI) species (i.e. *L. pallida*) would be cloned and interplanted with a different cross-compatible pollen donor species. This approach should encourage insect mediated cross-pollination and interspecific seed production on the SI clone. Although several workers (Brewbaker and Sorensson, 1990; Wheeler, 1991) endorsed this strategy, it has only been attempted once, with disappointing results (Bray and Fulloon, 1987). Bray and Fulloon (1987) cloned the SI *L. pulverulenta*, and interplanted it with pollen donors of *L. leucocephala*. Surprisingly, only 7%, 9% and 29% hybrid seed was produced over three consecutive years, leading them to conclude that the *L. pulverulenta* clone used was not entirely SI.

In an attempt to produce open-pollinated interspecific F1 hybrid seed we established three seed orchards in Hawaii. The effect of rootstock genotype on the vegetative growth in two to these orchards (orchards 1 and 2) was described in the accompanying paper (Brennan et al., 1998). The present paper reports on the analysis of the genotypes of seedlings from *L. pallida* K804 in orchard 2, and *L. pallida* K953 in the previously undescribed orchard 3, in order to determine if, and to what extent, the goal of hybrid seed production was achieved.

Materials and methods

Seed orchards

The two seed orchards were located on a farm on the Kona side of the island of Hawaii at 200 m a.s.l. The climate and soil conditions of the farm are described as orchard 2 in the accompanying paper (Brennan et al., 1998). In the present paper we analyze seedlings from trees of a single *L. pallida* K804 clone in orchard 2 (described in detail in Brennan et al., 1998), and trees of a single clone of *L. pallida* K953 in orchard 3 (described below). Due to pod abortion on the self-incompatible clones in orchard 1 we were unable to obtain seed for analysis of parentage. Orchards 2 and 3 were separated from each other by a 40 m strip of fruit trees to reduce the chance of cross pollination between the *L. pallida* clones. Although orchard 2 contained trees of both *L. pallida* clones (K804 and K953), only the K804 clone in this orchard flowered during our study; thus the K953 trees in orchard 2 are not further considered.

Orchard 3 was designed to produce F1 hybrid seed of *L. pallida* K953 × *L. leucocephala* K636. The *L. pallida* K953 clone was obtained from a single seedling grown in the green house at Cornell University from the University of Hawaii germplasm. From March 1993 to December 1994, the seed orchard trees were produced by grafting the various scion clones onto compatible seedling rootstocks in the greenhouses at Cornell University using the grafting methods described in Brennan and Mudge (1998). The orchard was planted

in November 1993 (eight months prior to orchard 2) using grafted *L. pallida* K953 trees, and *L. leucocephala* K636 seedlings grown in Hawaii. The orchard contained 17 grafted *L. pallida* K953 trees interplanted with 21 *L. leuco-cephala* K636 seedlings. The *L. pallida* K953 and *L. leucocephala* K636 trees were alternately planted in two rows at a 2 × 2 m spacing in the 7.5 m wide alley between existing hedge rows of 6–8 m tall, seed producing *L. leuco-cephala* K636 trees. It was intended that the *L. leucocephala* K636 hedge rows would donate pollen, and create a physical barrier to reduce cross-pollination from unintended pollen donors such as the weedy *L. leucocephala* K997 around the farm, and *L. pallida* K804 in orchard 2. Cardboard mulch was used for weed control, and the trees were drip-irrigated using an automated polyethylene system.

Flowering data and seed collection

The flowering status of all the trees in both orchards was monitored during the flowering season from June through August 1994, and in January 1995 all seed produced on the *L. pallida* clones was collected for subsequent analysis of parentage in greenhouses at Cornell University.

Seedling production

In February 1995, a random sample of approximately 50 seeds from the *L. pallida* K804 trees in orchard 2, and 100 seeds from *L. pallida* K953 trees in orchard 3 was scarified by nicking and germinated on filter paper in the dark. The seed was inoculated with the TAL 1145 *Rhizobium* peat mixture (Agroforester Tropical Seeds, Holualoa, Hawaii) and sown in D-pots filled with Metro-mix 360. Germination was approximately 50% for seed of both *L. pallida* clones presumably due internal seed damage by the koa haole seed beetle (*Araecerus livipennis*). Thus only 21 seedlings from *L. pallida* K804, and 45 seedlings from *L. pallida* K953 were obtained. The seedlings were grown in greenhouses under 400 watt HID lights set on a 13-hour day length. The greenhouse temperature was 23 to 27 °C during the day and 21 to 23 °C during the night.

Morphological analysis

Leucaena species have bipinnately compound leaves consisting of pinnules (sub leaflets) arranged on pinnae (leaflets), that are attached to the rachis (midrib). Previous studies (Bray and Fulloon, 1987; Sorensson and Shelton, 1992; Hughes and Harris, 1994) have used leaf characters as morphological markers to reliably determine hybridity in *Leucaena*.

In June 1995, when the seedlings were approximately 1 m tall, each seedling was evaluated for overall leaf appearance, petiolar gland shape and color, and the number of pairs of pinnule per pinna on the 4th through 15th

true leaf. Pinnule pairs per pinna were counted on one of the middle pairs of pinna of each leaf. Our pinnule/pinna data for all of the seedlings were compared with that of Sorensson and Shelton (1992). Their data were used since seedlings of the maternal species and possible pollen donors with the appropriate 4th through 15th true leaf were not available. Sorensson and Shelton (1992) obtained pinnule pairs per pinna data for seedling progeny from hand-pollinations between two accessions of *L. pallida* (K806 and K748) since the species is SI. Their data for the pollen donor was from one of the same pollen donors (*L. leucocephala* K636) used in our study. In comparing our data to those of Sorensson and Shelton, we assume that the environment where the seedlings were grown does not significantly influence the vegetative traits evaluated. This assumption is justified since comparisons of leaf measurements and morphology on our adult greenhouse-grown plants, corresponded well to measurements of adult field grown plants by Sorensson (1993), and Hughes and Harris (1994).

Pinnule width measurements were taken from the putative self-pollinated progeny (selfs) and putative interspecific hybrids of the *L. pallida* clones, their maternal parents and potential pollen donors. Six replicate seedlings of the putative selfs and hybrids from *L. pallida* K804 and *L. pallida* K953 were randomly chosen for pinnule width measurements. Using a dissecting microscope with a calibrated micrometer, pinnule width was measured on the middle pair of pinnules taken from the middle pair of pinna on the 11th true leaf. For each replicate seedling, the pooled average of the four pinnule widths was calculated. Leaves of greenhouse-grown specimens with a similar number of pinna/leaf were similarly evaluated for the maternal *L. pallida* clones (K804 and K953) and intended pollen donors (*L. leucocephala* (K420, K481, K636) and F1 KX3 (*L. diversifolia* K156 × *L. leucocephala* K636)). For two possible unintended pollen donors grown as rootstock controls (*L. diversifolia* K156 and *L. leucocephala* K997) in orchard 2, pinnule measurements were obtained from one year old, greenhouse-grown seedlings. Leaf samples of a third possible unintended pollen donor (diploid *L. diversifolia* K749) were not available; hence we obtained pinnule width measurements from another diploid line (*L. diversifolia* K907). Our estimation of pinnule width of *L. diversifolia* K749 from *L. diversifolia* K907 assumes little variation in pinnule width among various lines of diploid *L. diversifolia*; this assumption is justified since Sorensson (1993) found little variation in pinnule width within several lines of diploid *L. diversifolia*.

Statistical analysis

Analysis of variance (ANOVA) was conducted on the pinnule width measurements. Single degree of freedom contrasts were used to compare the pinnule width of the progeny with their maternal parent. The statistical analysis was done using the Proc GLM procedure in SAS (SAS Institute Inc., Cary, NC).

Results

Flowering synchrony and pod set

Table 1 shows the percentage of trees which flowered for each of the *L. pallida* clones, and the potential pollen donors in and around both seed orchards in August 1994. In orchard 2, 38% of the *L. pallida* K804 trees flowered in synchrony with 26% overall of the grafted (intended) pollen donor trees. Thus in this orchard the ratio of flowering *L. pallida* K804 to flowering intended pollen donors was 1:7. Several unintended possible pollen donors in orchard 2 also flowered including the rootstock controls (*L. diversifolia* K156 and *L. leucocephala* K997), and *L. diversifolia* K749. The *L. pallida* K804 trees were less than 1 m tall when they flowered and produced a total of 3 g of seed by January 1995. In orchard 3, 59% of the *L. pallida* K953 trees flowered in synchrony with all of the *L. leucocephala* K635 trees in the surrounding hedgerow pollen donors. The *L. pallida* K953 trees were several meters tall and seeded heavily on the distal 1 m of many shoots to produce 350 g of seed by January 1995.

Morphological analysis

In June 1995 (14 weeks after planting) there were two visually distinct groups of seedlings from each of the *L. pallida* clones. Figure 1 shows pinnule and pinna silhouette images (photocopies) of the *L. pallida* K953 maternal parent (Figure 1A), *L. leucocephala* K636 (Figure 2D; a likely pollen donor), and the two visually distinct categories of seedlings from seed harvested from

Table 1. Percentage of trees flowering during August 1994 in hybrid seed orchard 2 and orchard 3, near Kona, Hawaii, USA.

|		% trees flowering
Orchard 2		
Grafted trees	*L. pallida* K804	38
	L. pallida K953	0
	L. leucocephala K420	3
	L. leucocephala K481	36
	L. leucocephala K636	0
	F1 KX3 (*L. diversifolia* K156 x *L. leucocephala* K636)	57
Ungrafted trees	*L. diversifolia* K156 (rootstock control)	30
	L. leucocephala K636 (rootstock control)	0
	L. leucocephala K997 (rootstock control)	80
	L. diversifolia K749 (green manure)	100
Orchard 3	*L. palliada* K953 (grafted)	59
	L. leucocephala K636 (seedlings)	0
	L. leucocephala K636 (hedgerows)	100
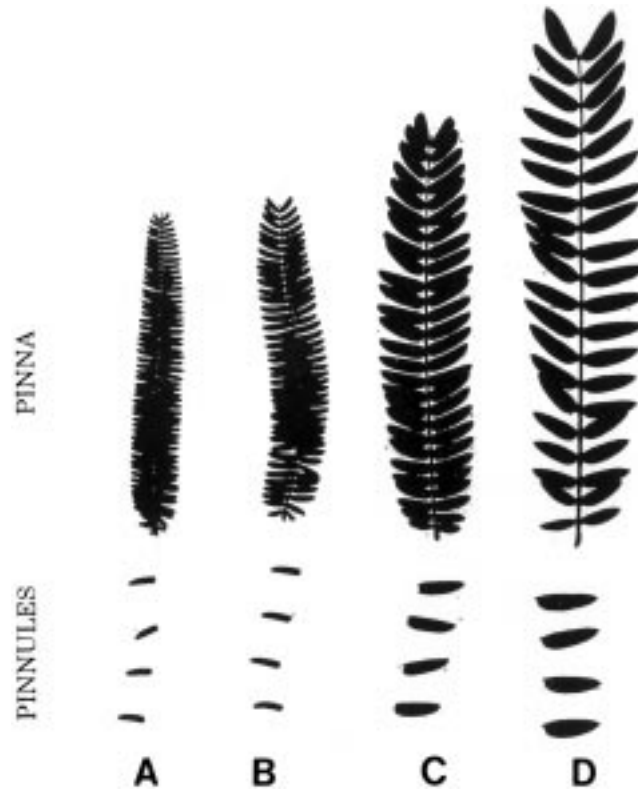
Figure 1. Sihouette of pinnules and pinna of *L. pallida* K953 (A), a *L. pallida* K953 putative selfed progeny (B), a *L. pallida* K953 interspecific hybrid progeny (C), and a likely pollen donor, *L. leucocephala* K636 (D). Images are approximately 50% of their actual size. Trees were grown in a greenhouse in Ithaca, NY, USA.

the *L. pallida* K953 orchard. Seedlings with the smaller pinna and pinnules (Figure 1B) resembled the maternal parent (*L. pallida* K953), in terms of pinnule pairs per pinna, pinnule size, as well as shape, color and depth of the petiolar glands. In contrast, seedlings with the larger pinna and pinnules (Figure 1C) had leaf traits intermediate to their maternal parent *L. pallida* K953 and possible pollen donor (*L. leucocephala* K636). Based on these traits, the group of seedlings (Figure 1B) resembling the maternal parent was tentatively judged to be self-pollinated progeny while the other group (Figure 1C) were considered to be interspecific hybrids. At this stage in their growth, both groups were vigorous (about 115 cm tall) and could not be differentiated on the basis of height.

Figures 2 and 3 show the number of pinnule pairs/pinna as a function of leaf number for all 21 of the *L. pallida* K804 and all 45 of the *L. pallida* K953 progeny, respectively. There is a strong linear relationship between the true leaf number and pinnule pairs per pinna for the *L. pallida* K804 ($r^2 = 0.84 -$
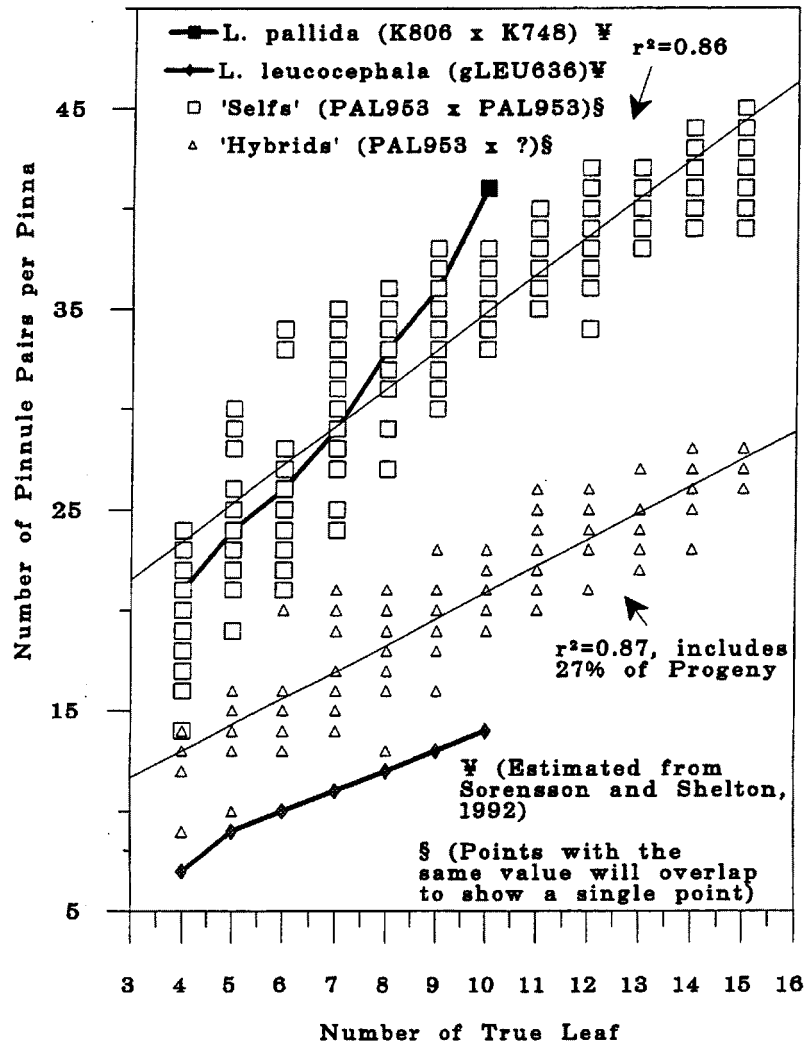
Figure 2. Pinnue pairs/pinna for *L. pallida*, K806 × K748 (■), *L. leucocephala* K636 (◆) and *L. pallida* K953 putative selfs (□) (r^2 = 0.86) and interspecific hybrids (△) (r^2 = 0.87, includes 27% of progeny). Data for *L. pallida* (K806 × K748), and *L. leucocephala* K636 are from Sorensson and Shelton (1992). Other data are from trees grown in a greenhouse in Ithaca, NY, USA. Overlapping points with the same value appear as a single point.

0.90), and the *L. pallida* K953 (and r^2 = 0.86 – 0.87) progeny. Figures 2 and 3 show two distinct groups of progeny including putative self-pollinated progeny, and interspecific hybrids. Clear differences between the progeny groups were evident by the 4th to 5th true leaf for *L. pallida* K804 (Figure 2), and the 7th and 8th true leaf for *L. pallida* K953 progeny (Figure 3). Based
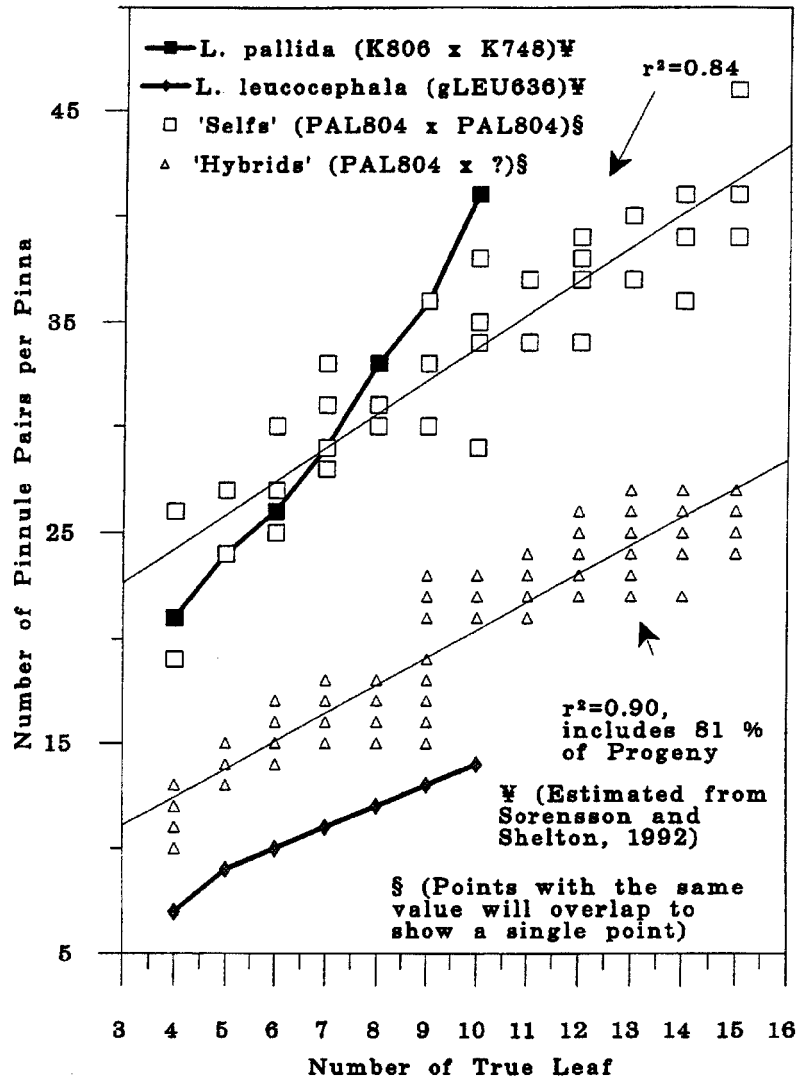
Figure 3. Pinnule pairs/pinna for *L. pallida*, K806 × K748 (■), *L. leucocephala* K636 (◆) and *L. pallida* K804 putative selfs (□) ($r^2 = 0.84$) and interspecific hybrids (△) ($r^2 = 0.90$, 81% of progeny). Data for *L. pallida* (K806 × K748), and *L. leucocephala* K636 are from Sorennson and Shelton (1992). Other data are from trees grown in a greenhouse in Ithaca, NY, USA. Overlapping points with the same value appear as a single point.

on these data, 81% of the progeny from *L. pallida* K804 were judged to be interspecific hybrids compared to 27% of the *L. pallida* K953 progeny.

Figure 4 shows the mean pinnule width for the *L. pallida* clones, their progeny, and potential pollen donors. The pinnule width data show one group
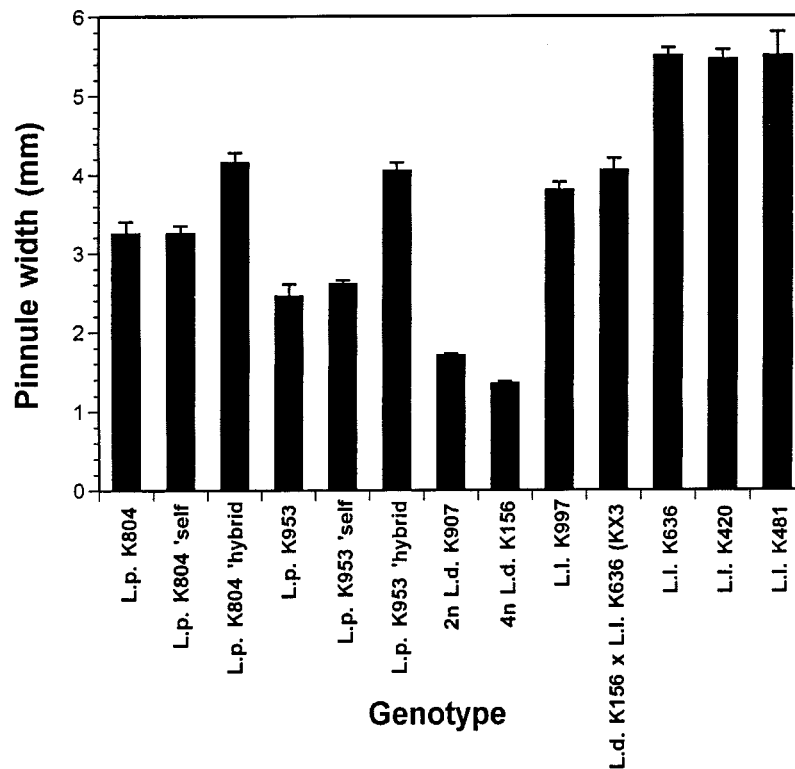
Figure 4. Mean pinnule width of *L. pallida* clones (K804 and K953) and their progenies, and potential pollen donors in the area. Measurements are from trees grown in a greenhouse in Ithaca, NY, USA. L.1., L.p., L.d. refer to *L. leucocephala*, *L. pallida*, and *L. diversifolia*, respectively.

of the seedlings (putative selfs) from *L. pallida* K804 and *L. pallida* K953 that are nearly identical to their maternal parent, and another group (interspecific hybrids) that are between the maternal parent and three *L. leucocephala* accessions (K420, K491, K636). There were no significant difference between the pinnule width of either maternal parent and the putative self-pollinated seedlings (Table 2). However, the interspecific hybrid progeny had significantly greater pinnule width than their respective maternal parent ($P < 0.001$).

Discussion

Based on our data of pinnule width and pinnule pairs per pinna, both *L. pallida* clones produced interspecific hybrid seed in open-pollinated seed orchards. However, the percentage of interspecific hybrid seed produced depended on the maternal *L. pallida* clone since *L. pallida* K804 produced 81% inter-

Table 2. ANOVA for pinnule width of *L. pallida* (K804 and K953), their progeny, and potential pollen donors from hybrid seed orchards near Kona, Hawaii, USA.

Sources	df	Mean squares	Significance
Genotype[a]	12	10.96	****
Contrasts			
K804 vs K804 Selfs	1	0.0008	ns (0.93)
K953 vs K953 Selfs	1	0.0690	ns (0.45)
K804 vs K804 Hybrids	1	2.3964	****
K953 vs K953 Hybrids	1	7.8408	****
K804 vs K953	1	1.8291	****
Error	65	0.1109	
Total	77		

[a] Genotypes included are *L. pallida* K804 and progeny, *L. pallida* K953 and progeny, 2*n L. diversifolia* K907, *L. diversifolia* K156, *L. leucocephala* (K420, K481, K636, K997), and the F1 hybrid KX3 (*L. diversifolia* K156 × *L. leucocephala* K636).

ns is not significant and is followed by the actual *P* value.

**** is significant at the $P < 0.0001$ level.

specific hybrid seed compared to only 27% produced by *L. pallida* K953. It appears that the most likely pollen donors of the interspecific hybrid seed produced in either seed orchard were *L. leucocephala* (K420, K481, K636) (Figure 4). Based on pinnule pairs per pinna data, the interspecific hybrids produced in both orchards closely resemble interspecific hybrids produced from hand-pollination between various *L. pallida* lines and *L. leucocephala* K636 (Sorrensson and Shelton, 1992). Considering the abundance of flowering weedy *L. leucocephala* K997 in the area surrounding these orchards (and as rootstock controls in the *L. pallida* K804 orchard), it is encouraging that K997 apparently did not pollinate either *L. pallida* clone. The vigorous upright growth of all the interspecific hybrid seedlings further confirms that weedy *L. leucocephala* K997 was not a pollen donor, since F1 hybrid progeny from hand pollination's between *L. pallida* and *L. leucocephala* K997 have stunted growth relative to hybrids fathered by other *L. leucocephala* (K420, K481 and K636) (Pers. Comm., W. Sun; pers. obser. Brennan; 1993).

There are three possible scenarios that may explain the parentage of the *L. pallida* progeny group with leaf characteristics similar to their maternal parent including (1) self-pollination by the *L. pallida* clones, (2) intraspecific pollination (i.e. *L. pallida* K804 × *L. pallida* K953, and the reciprocal), and (3) interspecific pollination by another *Leucaena* species with similar leaf characteristics to *L. pallida*. The least likely scenario is the third since, as Figure 4 shows, there are not any other flowering *Leucaena* species in or near the orchards with a similar pinnule width to either *L. pallida* clone. The second scenario of cross pollination between the *L. pallida* K804 in orchard 2 and *L. pallida* K953 in orchard 3, although possible, is unlikely for several reasons. If the *L. pallida* clones had crossed we would expect progeny with

a pinnule width that is intermediate to that of the maternal clones. No such group was apparent from the results shown in Figure 4. In addition, it is unlikely that honeybees cross pollinated *L. pallida* K804 and *L. pallida* K953 in the separate orchards since studies with apples have shown that honey bees usually move within a row while foraging rather than between rows (Mayer et al., 1989). Furthermore, *L. pallida* K953 trees produced more than 100 times the number of seed and flowers than *L. pallida* K804 trees, therefore if the two clones crossed we would expect fewer interspecific hybrids in the progeny of *L. pallida* K804 than the *L. pallida* K953 progeny. The data, however, show the opposite (Figures 2 and 3). The most likely scenario to explain the parentage of the progeny group with leaf characteristics similar to their maternal parent is self-pollination due to a break down of the self-incompatibility mechanism. The comparisons of pinnule width of the maternal clones with the putative selfs show no significant difference, thus supporting our conclusion that self-pollination occurred (Table 2).

There are several natural conditions which can cause the breakdown of gametophytic self-incompatibility including (1) delayed pollination where fresh pollen can fertilize over-mature stigmas, (2) end-of season effects where late season flowers are self-compatible but early season ones are self-incompatible, (3) mentor effects where self-incompatible pollen becomes compatible when mixed with foreign pollen, and (4) high temperature effects where high temperatures remove the self-incompatibility mechanism (Richards, 1986). It is possible that any of these conditions occurred in our seed orchards to allow self-pollination within either *L. pallida* clone. If the seed orchard system used here is to become a feasible means of producing large quantities of F1 interspecific hybrid seed, it is necessary to determine which of these conditions can cause self-incompatibility to break down in reportedly SI species of *Leucaena*. By understanding which conditions favor SI break down, the seed orchard manager may be able to manage the orchard and harvest seed which is most likely to be the result of interspecific hybridization.

With insect pollinated crops like cotton (Waller and Mamood, 1991) and *Capsicum* (Daskolob an Mihailov, 1988), cytoplasmic male sterile (CMS) lines have been used to produce virtually 100% F1 hybrid seed by insect-mediated cross-pollination between a CMS line and a pollen donor line. Although the SI mechanism in *Leucaena* is different from CMS, they function similarly to promote outcrossing, and thus we expected nearly 100% F1 interspecific hybrid seed set on the *L. pallida* clones. Our results and those of Bray and Fulloon (1987) indicate significant self-pollination in *L. pallida* and *L. pulverulenta*, although both species are reported to be SI. Although the *L. pallida* K804 produced more interspecific hybrid seed than *L. pallida* K953 in the first year of production, more years of seed production data are required to conclude that either clone is more suitable in open-pollinated interspecific hybrid seed production.

Sorensson and Brewbaker (1994) reported 'rare selfed progeny' produced on several species considered SI (most diploids and *L. pallida*), as a result of

interspecific hand-pollination. Hand-pollination has been commonly used in *Leucaena* to determine compatibility (Sorensson and Brewbaker, 1994). There are several reasons why insect-pollination could result in selfing where hand-pollination may not. Insect pollinators are more persistent, frequent and efficient at self-pollinating than even the most skilled breeder is. For example, a breeder will normally attempt to self pollinate a flower once and then isolate it in a bag (Sorensson, 1988). In contrast, several individuals and possibly species of insects may repeatedly visit a receptive flower throughout its lifetime, with pollen sources from several other flowers from the same clone. It may be possible to select clones of SI species where SI is stable or breaks down infrequently, and use these in open-pollinated interspecific *Leucaena* hybrid seed production. With alfalfa which is also gametophytically SI, breeders have noted significant variation in the stability of SI and have succeeded in selecting clones that produce stable, high levels of hybrid seed and may be useful in hybrid seed production (Cambell and Bauchan, 1990).

Although the seed produced on the *L. pallida* clones in our study were not 100% inerspecific hybrids, it was relatively easy to distinguish the hybrids from the selfs based on the overall appearance and/or pinnule characteristic. Previous studies with *Leucaena* species (Bray and Fulloon, 1987; Sorensson and Shelton, 1992) classified progeny as selfs or hybrids based on overall appearance, as well as pinnule widths and number. Hughes and Harris (1994) were able to differentiate hybrids from selfs using either molecular DNA techniques or morphological characters. This confirms that inexpensive, simple and rapid morphological measurements (pinna/leaf, pinnules/pinna, etc.), and even overall appearance, are adequate for determining hybridity and can be used with confidence. The hybrid seed producer might adjust the price of seed based on the estimated percentage of selfs, and provide the buyer with simple instructions on how to identify these based on morphological traits. Although the pollen donor is known to affect seed weight and size of some species (Anderson, 1990; Kahn et al., 1994), maternal effects are generally much larger (Molau, 1991). Attempts to distinguish selfed and cross-pollinated seeds based on size or weight in large-scale hybrid seed production have generally failed (Kilen, 1980; Bray and Fulloon, 1987).

Conclusion

Interspecific F1 hybrid seed was successfully produced on two different clones (K804 and K953) of *L. pallida* in separate orchards during the first year of production. Evidence for considerable selfing of the *L. pallida* clones, strongly suggests that a break down of the gametophytic self-incompatibility mechanism frequently occurred. Research to determine the conditions that cause the break down of self-incompatibility in *Leucaena* is required.

Acknowledgements

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