Breeding behaviour of *Santalum lanceolatum* self-, intra- and interspecific cross-compatibility

Tony Page and Hanington Tate
School of Marine and Tropical Biology, James Cook University, Cairns, Queensland 4870

S. album

S. lanceolatum

S. austrocaledonicum

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Contents

1 Abstract

Controlled pollination using 13 genotypes of *Santalum lanceolatum* was undertaken to elucidate (i) self-incompatibility (ii) intraspecific cross-compatibility in the species, and (iii) interspecific cross-compatibility with *S. album* and *S. austrocaledonicum*.

Santalum lanceolatum may be considered to have a facultative allogamous (incomplete outbreeding) breeding system. This study found variation between genotypes in the level putative self-incompatibility, where some (20%) were found to set seed following self-pollination, while the remaining 80% had no seed development with such pollinations. However, a significantly greater proportion of genotypes, developed seed following intraspecific cross pollination (62%) compared with self-pollination (20%). In accession 2 where sufficient self and cross pollinations were performed no significant difference were found between them percentage seed set. The seed set from self-pollination were successfully germinated and have been growing for 2 years without any substantial morphological distinction between inbred and outcrossed seedlings.

While total geographic isolation and significant morphological divergence exists between *S. lanceolatum* with each of *S. album and S. austrocaledonicum* this study found no indication of reproductive barrier(s) between them. No significant differences were found in the percentage seed set among *S. lanceolatum* intraspecific crosses (7.5%) compared with reciprocal *S. lanceolatum* x *S. austrocaledonicum* interspecific crosses (7.6%). Germination of seed derived from intraspecific outcross pollinations was found to be low (41%) relative to interspecific pollinations with each of *S. album* (114% - with many seeds producing two seedlings) and *S. austrocaledonicum* (70%). Therefore while seed set from intraspecific outcross pollinations was greater than for reciprocal *S. lanceolatum* x *S. album* crosses (4.3%), no significant differences were found for the percentage of seedlings developed from these two pollination types (2.5% and 4.8% respectively).

The results of this study have implications for both the domestication of *S. lanceolatum* for its commercial production and for conservation of its natural stands. The use of genetic variation present within the high quality *S. album* and *S. austrocaledonicum* could be used for the improvement of *S. lanceolatum* and vice versa. However, inappropriate planting of foreign each of these species within the their natural ranges is likely to result in gene exchange among them and affect the genetic integrity of these natural populations.

2 Introduction

Santalum (sandalwood) is a genus of hemi-parasitic tree species occurring throughout south and south-east Asia, Australia and the Pacific. The heartwood of several species produces valuable aromatic oil widely used in perfumery, medicines and incense. Throughout the world, sandalwood products are being sourced from declining natural stands and the international price for natural sandalwood products continues to increase. Therefore significant opportunity exists to establish commercial sandalwood agroforests, to reduce pressure on wild stands, improve consistency of product supply and increase economic outcomes for smallholder farmers.

In Queensland, sandalwood (*S. lanceolatum*) has long been commercially exploited for its powdered heartwood used in funeral pyres and insence. Harvesting natural sources of sandalwood in Cape York commenced after 1900 and continued until the early 1930's, which was stimulated mainly by demand from China. Many of the European sandalwood-getters relied heavily on the local knowledge and labour of Aboriginal people in each area to find and harvest the trees (Wharton 2005). In Queensland the indigenous sandalwood (*S. lanceolatum*) has been exported to Asia as wood from Cape York Peninsula since the late 19th century.

While little commercial harvesting continues on Cape York today because of the scattered resource, indigenous communities are interested in re-establishing the sandalwood resource to support local enterprise. The lower quality of *S. lanceolatum* oil compared with other commercial sandalwood species such as *S. album*, *S. yasi* and *S. austrocaledonicum* has limited the commercialisation of this species as cultivated sandalwood. However with recent identification of high quality *S. lanceolatum* in Cape York (Page *et al.* 2007) there is opportunity to develop this resource for commercial agroforestry plantings.

The development of *S. lanceolatum* as significant agroforestry crops will depend on the development of forms suited to commercial production, with high growth rates yielding high volumes of heartwood containing concentrated oils with high levels of α- and β-santalol. The implementation of a successful breeding programme for any sandalwood species will depend upon knowledge of its breeding system and its cross-compatibility with related species that are a source for potentially useful characters. Given also the continued exploitation of *S. lanceolatum* in Queensland a knowledge of its breeding system will assist those developing strategies aimed at conserving current wild populations and establishing new plantings within it natural distribution. Information on the breeding system and patterns of gene flow are important for planning germplasm collection, designing and managing seed orchards and for

maintaining genetic diversity in breeding populations The objectives of the present study were to determine levels of (i) self- and (ii) cross-compatibility within *Santalum lanceolatum, S. album* and *S. austrocaledonicum* and (iii) cross-compatibility between these three sandalwood species.

3 Materials & Methods

3.1 Stages of flower and inflorescence development

Phenological stages of flowers and inflorescences of *S. lanceolatum, S. album and S. austrocaledonicum* were determined by twice daily observations of an individual inflorescence from 3 accessions of each species. These observations were undertaken from the period of anthesis of the first flower, to petal fall and style desiccation of the last flower on a given accession. Based on these observations, phenological stages in the development of a single flower were identified. A single inflorescence from each species was photographed daily. Phenological stages of a flower were measured relative to the day of anthesis, or the time of flower-opening, which was considered to be day zero on its development scale.

3.2 Controlled pollination

Grafted clones of *S. lanceolatum*, *S. album* and *S. austrocaledonicum* were grown in 300mm-diameter pots in a soil-less potting medium in an insect-proof greenhouse with drip irrigation. Flowers were emasculated during anthesis using pointed forceps. The anthers removed during this process were either placed in small plastic vials and placed in a desiccator with silica gel or used immediately for pollination. All pollinations were made using pollen collected during the day (i.e. pollen was not stored and used on subsequent days). Pollinations were carried out by applying the pollen-shedding anther to the stigma until pollen grains had adhered to the stigma. Individual inflorescences were pollinated with a single pollen source and each was tagged with details of pollen donor.

Thirteen genotypes of *Santalum lanceolatum* (accessions 0, 1, 2, 5, 8, 10, 14, 16, 25, 27, 29, 30 and 31) collected from Cape York Peninsula were used to examine self-incompatibility within, and intraspecific compatibility between them. Eleven of these genotypes (accessions 0, 2, 5, 10, 14, 16, 25, 27, 29, 30 and 31) were used also in crosses to evaluate the reciprocal interspecific compatibility with three genotypes of *S. album* (E5, E7 and E8). Five *S. lanceolatum* genotypes (accessions 2, 5, 14, 16, and 29) were used in reciprocal crosses with one genotype of *S. austrocaledonicum* (T1). Seed production in *S. lanceolatum* was examined in 182 unpollinated, 232 self-pollinated, 241 outcross (between different accessions) 1486 interspecific (1250 with *S. album* and 236 with *S. austrocaledonicum*) pollinated flowers (Table 1). Genotype

combinations and unique pollinations are pollination treatments involving a unique combination of genotypes. For instance a cross between accessions 1 and 2 would be considered to be unique from a cross between accessions 1 and 5.

Pollination Type	Genotype Combinations	Flowers 'Treated'
S. lanceolatum unpollinated	7	182
S. lanceolatum self-pollinated	10	234
S. lanceolatum intraspecific	13	241
S. lanceolatum x *S. album*	20	820
S. album x *S. lanceolatum*	23	430
S. lanceolatum x *S. austrocaledonicum*	5	116
S. austrocaledonicum x *S. lanceolatum*	5	120
Total	**83**	**2143**

Table 1: The number of genotype combinations (unique 'pollinations') and treated/pollinated flowers for seven different pollination types.

Pollinations were carried out on three separate flowering events during September 2007, December 2007 and February 2008. Flowers were left on the plants for approximately 8-10 weeks from pollination to fruit harvest. Fruits from each pollination category were collected, the flesh was removed and the seed air-dried before storing in a sealed plastic containers at 4°C.

Germination of seed resulting from controlled pollination was undertaken in a seed raising mix with a 1:1 ratio of medium grade perlite and vermiculite. Seeds were placed under 50% shade and were watered through an automatic irrigation system for 15 minutes per day. Seeds were considered germinated after they had been pricked into pots and survived for a period of 3 months.

Differences in the (i) proportion of pollinated flowers developing into seed and seedlings between pollination types (i.e. unpollinated, self-pollinated, intraspecific out-cross pollinated etc.) and (ii) the proportion of unique pollinations developing seed and seedlings were evaluated using an equality test of two binomial proportions (Ott and Longnecker 2001) calculated by:

$$z = \frac{(\hat{\pi}_1 - \hat{\pi}_2)}{\sqrt{\frac{\hat{\pi}_1(1-\hat{\pi}_1)}{n_1} + \frac{\hat{\pi}_2(1-\hat{\pi}_2)}{n_2}}}$$

The two binomial populations are denoted by $\hat{\pi}_1 = \frac{y_1}{n_1}$ and $\hat{\pi}_2 = \frac{y_2}{n_2}$ where y_1 seeds/seedlings are recorded for the random sample of n_1 pollinations from population 1, and y_2 seeds/seedlings are recorded for the random sample of n_2 pollinations from

population 2. The null hypothesis was rejected where the absolute value of the statistic z was greater than $z_{0.05} = 1.645$.

This statistical approach was used because, although a sufficient number of pollinations per pollination type were performed, in some cases a low number of replicates or genotype combinations did not permit evaluation by two-way ANOVA.

4 Results

4.1 Flower and inflorescence development in *Santalum lanceolatum*

Flowers of *S. lanceolatum* are found within terminal or axillary panicles consisting of greater than 10 flowers. Individual flowers open and close within 12-24 hours, often opening in the morning and closing by the evening of a given day. All flowers on the inflorescence can complete their opening and closing over a period of 7-14 days, depending upon prevailing weather conditions.

The floral tube is approximately 3mm long and the anther filaments, hypanthial lobes and tepals emerge from the top. The corolla consists of 4-, rarely 5-tepals, which together with the anthers, alternate with the hypanthial lobes. The width of the flower the tips of each tepal ranges from 5-7mm. Sweet smelling nectar is produced at the base of the floral tube.

Anther filaments are short (1.0-1.5mm long) and dorsifixed to anthers (1.5-2.0mm long) that shed pollen along longitudinal slits. Trichomes (0.5-1.5mm long) are found at the base of each anther filament extending in the floral tube and towards the back of the anthers. Hypanthial lobes are typically yellow and alternate between anthers ranging from 1.0-1.5mm long

The style is approximately double the length of the floral tube and the stigma is presented approximately 0.5-1.0mm above the top of the anthers. The stigma has 3 to 4 lobes and short papillae, but no change in stigma morphology such as swelling, colour change or evidence of exudate was observed during the experiment. The ovary is inferior to the floral tube and once fertilisation has been effected, the floral tube is abscised from the pedicel. The ovary swells to become a single seeded drupe, and the floral tube abscission scar is observed at the top of the fruit. The flower abscission occurs rapidly (1-2 days) in cases where no fertilisation has taken place.

Phenological stages of *S. lanceolatum* may be simply described as opened or closed given that they perform this within 12-24 hours, with little morphological changes in

the reproductive structures during this brief period. Pollen shed occurs simultaneously as the flower opens (defined as anthesis) and, in the absence of sufficient pollinators, the flower can close again with apparently viable pollen remaining on the anthers. It is unclear whether the stigma is viable when the flower is open, but given that flower abscission in the absence of pollination occurs within 48 hours of closing, stigma receptivity is likely to be during the open phase or just after the flower closes. The timing of controlled pollination obviously needs to occur during the day of opening.

4.2 Flower and inflorescence development in *Santalum album*

While the general floral morphology of *Santalum album* is similar to that of *S. lanceolatum*, the flowers of *S. album* (a) have a longer 'life' – 7-9 days compared with 12-24 hours, (b) change colour from white to red rapidly after opening, (c) have a smaller and less prominent stigma that do not extend beyond the height of the stamens (d) have less trichomes at the base of the anther filament, particularly within the floral tube (e) have tepals that open but do not close.

In the inflorescences evaluated the mean number of days from flower opening to closing was 8.7 days, with the flower tepals opening white and remaining this colour for only a brief period (0.8 days) before gradually changing over 1.4 days from white to red and remaining red for the remaining 6.5 days before either the flower or floral tube is abscised.

The floral tube is approximately 2.5mm long and the anther filaments, hypanthial lobes and tepals emerge from the top. The width of the flower from the tips of each tepal ranges from 5-6mm when the flower first opens, but as the colour of the tepals change from white to red the tips recurve downwards.

Anther filaments and anthers are shorter than for *S. lanceolatum* (~1.0mm and 1.5mm long respectively). Trichomes (0.5-1.5mm long) are found at the base of each anther filament extending only towards the back of the anthers. Trichomes at the base of the anther filaments are either minute or absent within the floral tube. Hypanthial lobes are red from flower opening are shorter than in *S. lanceolatum* (0.5-1.5mm long).

The style is approximately 3mm long and the stigma is presented at, or slightly below, the level of the top of the anthers (approximately 0.5mm away from each anther) when the flower opens. As the flower develops the anthers recurve away from the stigma, increasing the distance between the stigma and anther to approximately 1.0mm. The stigma has 3 to 4 lobes and short papillae, the stigma and style change colour from white at opening to pink concurrently with the colour change in tepals.

No evidence of stigma exudate in *S. album* was observed during the experiment. The ovary is inferior to the floral tube. Number of flowers per inflorescence ranges from 20-40 flowers.

4.3 Flower and inflorescence development in *Santalum austrocaledonicum*

The general floral morphology of *Santalum austrocaledonicum* is similar to that of *S. lanceolatum* with very few distinguishing features between the two. The main difference between these species is the longer flower life of *S. austrocaledonicum* which have flowers that open and close within a 24-48 hour period compared with a 12-24 hour period for *S. lanceolatum*. A flower from *S. austrocaledonicum* will typically open during the morning of a given day and close again during the afternoon of the following day. The tepals of *S. austrocaledonicum* open more completely than *S. lanceolatum* where the tips of its tepals recurve downward within 12 hours of opening before closing again 36 hours later.

During observations of floral morphology and undertaking controlled crosses with all three *Santalum* species, it was evident that substantial variation in pollen production could be found among the accessions. Those with higher pollen production (Accessions 02 25, 29, E5 and T1) were typically more successful in siring seeds when used as a male parent. Interestingly these were also relatively more successful seed bearing parents. This apparent variation in fecundity could explain the existence of the folklore 'man' and 'woman' varieties reported for *S. austrocaledonicum* in Vanuatu (Siwatibau *et al.* 1998) and *S. macgregorii* in Papua New Guinea (Gunn *et al.* 2002). Further investigations of this breeding behaviour through replication of ramets is required, since this result may have been due to variation in the maternal resources, which resulted in lower general productivity of some compared with other accessions.

4.4 Unpollinated flowers

No signs of fruit development were observed in any of the unpollinated flowers in this experiment. Flowers of all species in this treatment were shed towards the end of their expected 'life' (*S. lanceolatum* 12-24 hours, *S. album* 7-9 days and *S. austrocaledonicum* 24-48 hours). No floral-tube abscission, indicating fruit development, was observed and no seeds were set from any flowers of this treatment.

4.5 Self-incompatibility in *S. lanceolatum*

Mean seed set per self-pollinated flower was 1.3%, which was significantly ($P<0.05$) fewer than the 7.5% of flowers in intraspecific cross-pollination (Figure 1). Seed set

following self-pollinations occurred in accession 2 and 29 where 3.6 and 7.4% of self-pollinated flowers set seed from 55 pollinations combined. The percentages for accession 2 were not significantly ($P<0.05$) different from intraspecific cross-pollinations involving this accession (used both as pollen donor or recipient) where 8.6% flowers set seed from 105 pollinations. No intraspecific cross-pollinations were performed using accession 29 so a similar comparison between self- and intraspecific cross-pollinations for this genotype was not possible. No seeds were set from any of the remaining 8 genotypes after a total of 179 self-pollinations.

Two of ten self-pollinated genotypes (20%) set seed in this experiment, which was significantly ($P<0.05$) lower than intraspecific pollinations, where 8/13 (62%) unique crosses developed seed (Figure 2). Likewise the percentage of unique 'crosses' developing seed within reciprocal interspecific hybridisations between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum* were significantly ($P<0.05$) greater (45% and 90% respectively) when compared with self-pollinated flowers. A similarly low-level of self-pollinated genotypes developed seedlings relative to interspecific crosses, but no significant difference was found between self- and intraspecific cross-pollinations (Figure 2).

The percentage of self-pollinated *S. lanceolatum* flowers developing into seed was significantly lower than for all other pollination types. The percentage of self-pollinated flowers that developed into seedlings however, was not significantly different from intraspecific crosses among *S. lanceolatum* genotypes and also between *S. album* (♂) and *S. lanceolatum* (♀) and S. lanceolatum (♂) and S. austrocaledonicum crosses (♀). Significantly greater percentage of flowers developing into seedlings were found for each of the interspecific crosses *S. lanceolatum* (♂) x *S. album* (♀) and S. austrocaledonicum (♂) x *S. lanceolatum* (♀) compared with *S. lanceolatum* self-pollinated flowers.

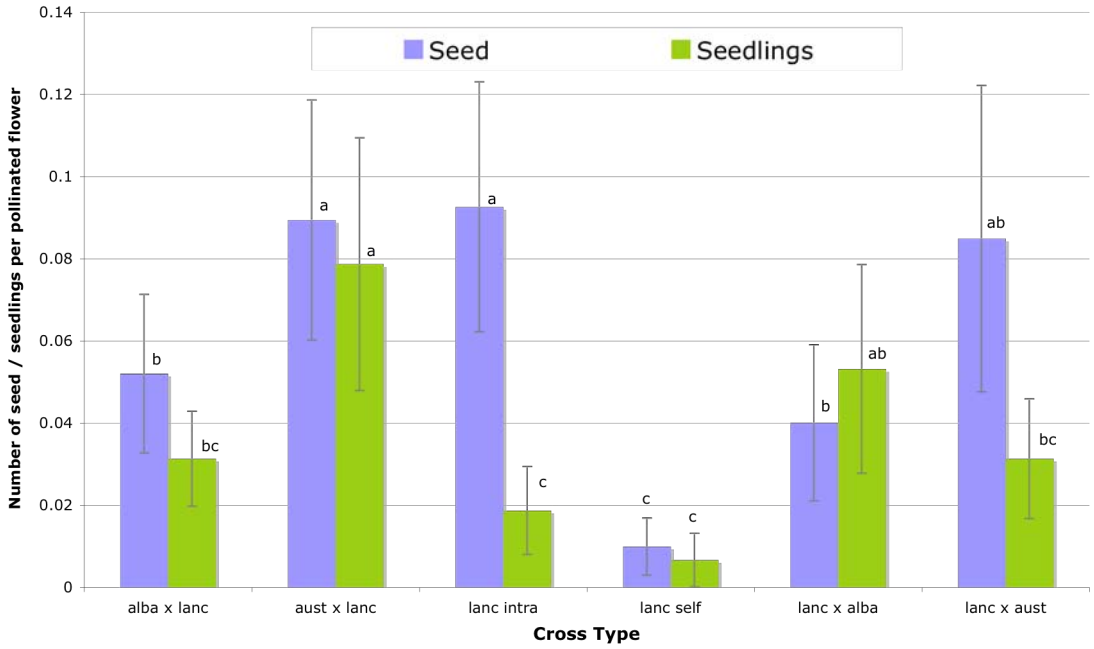

Figure 1: Number of seed and seedlings per pollinated flower for self and intraspecific pollinations in *S. lanceolatum* (lanc. self and lanc. intra respectively) and reciprocal interspecific pollinations between *S. lanceolatum* with each of *S. album* (lanc. x alba. and alba. x lanc.) and *S. austrocaledonicum* (lanc. x aust. and aust. x lanc.). Vertical bars represent standard errors. Cross types sharing lower case letters are not significantly (P<0.05) different within either the seed or seedling response variable.

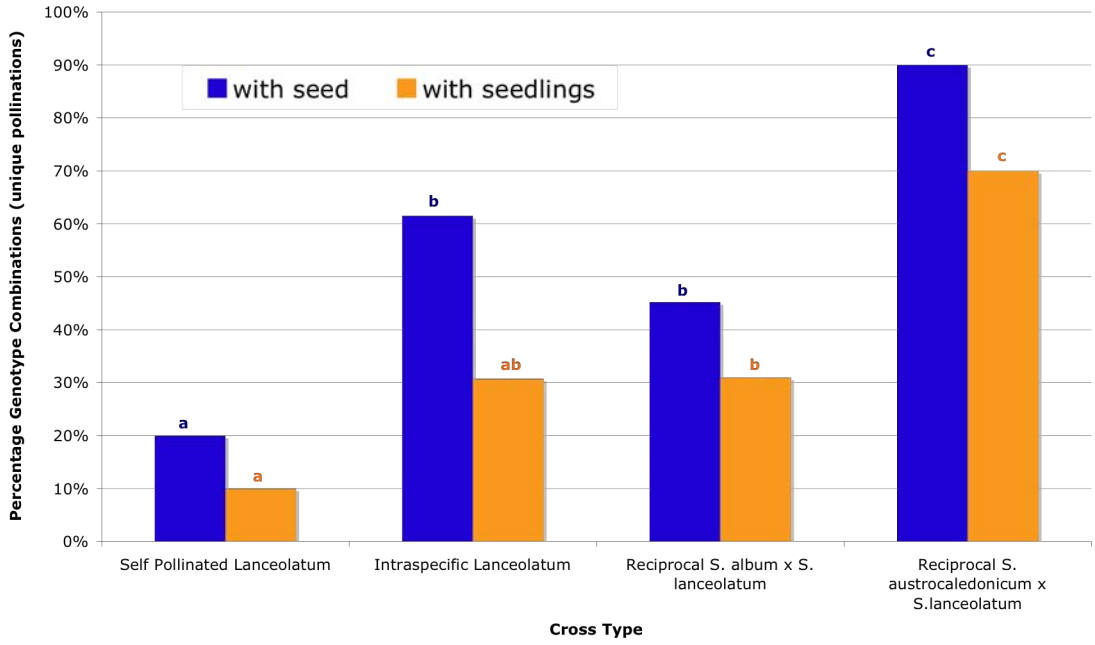

Figure 2: Percentage of unique pollinations (i.e. different self pollinated genotypes or different genotype combinations among cross types) with viable seed and seedlings. Cross types sharing lower case letters are not significantly (P<0.05) different within either the seed or seedling response variable.

4.6 Intraspecific cross-compatibility in *S. lanceolatum*

Of the 241 intraspecific crosses made in *S. lanceolatum* only 7.5% and 2.5% of pollinations resulted in the production of seed and seedlings respectively. For those crosses representing greater than 10 pollinations the seed set ranged from 0% in 3 different genotype combinations (averaging 16 pollination for each) to 14.2% in crosses between accessions 16 (♀) and 29(♂) (totalling 14 pollinations).

Only accession 25 was used in over 50 intraspecific cross-pollinations each as a pistillate and pollen parent with at least 3 different genotypes. The mean percentage of seed set per pollination in this accession was not significantly different between pistillate (4.8%) and pollen (5.4%) parent. No other accession had a sufficient number of pollinations or was crossed with at least 3 different genotypes to permit such evaluation of differences in fecundity between its use as either a 'female' or 'male' parent for intraspecific crosses.

While the number of seed developed per pollinated flower was significantly ($P<0.05$) greater in *S. lanceolatum* intraspecific crosses compared with self pollination, there was no difference among these cross types for the number of seedlings per pollinated flower (Figure 1). A similar pattern was found between these two cross types for the percentage of unique pollinations that developed seed where intraspecific crosses were significantly ($P<0.05$) greater but no statistical differences were found between these cross types for the percentage of unique crosses developing seedlings (Figure 2). No significant differences were found for unique crosses developing seed or seedlings between *S. lanceolatum* intraspecific and reciprocal *S. lanceolatum* x *S. album* interspecific. In contrast, a significantly ($P<0.05$) greater number of unique crosses were found to develop seed and seedlings in reciprocal *S. lanceolatum* x *S. austrocaledonicum* compared with *S. lanceolatum* intraspecific crosses (Figure 2).

4.7 Interspecific cross-compatibility between *S. lanceolatum* with *S. album*

Variation among the interspecific crosses between *S. lanceolatum* (♂) and *S. album* (♀) was found in the percentage of seed set per pollinated flower, ranging from 0–23% and from 0-16% in its reciprocal crosses (*S. album* (♂) and *S. lanceolatum* (♀)) for those crosses with greater than 10 pollinations. Interestingly 38% of the seeds developed from the former interspecific cross type resulted in 2 seedlings following germination. In crosses involving *S. album* (♂) and *S. lanceolatum* (♀) the percentage of seed producing 2 seedlings was 7.5%. No other cross type (self,

intraspecific or *S. lanceolatum* x *S. austrocaledonicum*) had seed that produced 2 seedlings.

A significantly (P<0.05) greater number of seeds per pollinated flower was found following intraspecific pollination among *S. lanceolatum* genotypes compared with reciprocal interspecific crosses between *S. album* and *S. lanceolatum*. However no significant differences were found in the number of seedlings per pollinated flower was found between crosses among *S. album* (♂) x *S. lanceolatum* (♀) and *S. lanceolatum* intraspecific pollinations. Furthermore crosses among *S. lanceolatum* (♂) x *S. album* (♀) had a significantly (P<0.05) greater number of seedlings per pollinated flower than for *S. lanceolatum* intraspecific pollinations.

Significantly (P<0.05) fewer unique crosses were found to develop seed and seedlings in reciprocal *S. lanceolatum* x *S. album* interspecific *compared with reciprocal S. lanceolatum* x *S. austrocaledonicum* crosses (Figure 2).

4.8 Interspecific cross-compatibility between *S. lanceolatum* with *S. austrocaledonicum*

In the present experiment only one genotype of *S. austrocaledonicum* (T1) flowered during the period of controlled pollinations. The flowering of this genotype coincided only with the flowering of five genotypes of *S. lanceolatum* (accessions 2, 5, 14, 16, and 29). Therefore in the evaluation of the compatibility between *S. lanceolatum* and *S. austrocaledonicum* only reciprocal crosses between T1 with each of accessions 2, 5, 14, 16, and 29 were possible.

Variation among the crosses between *S. lanceolatum* (♂) and *S. austrocaledonicum* (♀) was found in the percentage seed set per pollinated flower, ranging from 4–23% and from 0-18% in the reciprocal cross (*S. austrocaledonicum* (♂) and *S. lanceolatum* (♀)). No significant differences in the number of seed per pollinated flower were found between *S. lanceolatum* intraspecific crosses and each of the reciprocal interspecific crosses between *S. lanceolatum* and *S. austrocaledonicum*. Number of seedlings per pollinated flower for *S. austrocaledonicum* (♂) x *S. lanceolatum* (♀) cross was significantly (P<0.05) greater than both self- and intraspecific crosses within *S. lanceolatum*. The reciprocal interspecific cross (*S. lanceolatum* (♂) and *S. austrocaledonicum* (♀)) however, was not found to differ from these self- and intraspecific crosses.

5 Discussion and Conclusions

5.1 Stages of flower development

Morphological and phenological similarities between the latter two species suggest that similar natural pollinators may be responsible for affecting pollination in these species. The substantially greater longevity of individual *S. album* flowers compared with both *S. austrocaledonicum* and *S. lanceolatum* is an important phenological distinction between them.

The onset and duration of stages in the floral development in *S. album* was found to vary substantially between flowers on an individual. The rate of flower development, in *E. regnans,* can vary between flowers and seasons within a genotype, which may be strongly influenced by mean daily temperature (Griffin and Hand 1979). It is likely that stigma receptivity in *S. album* occurs during the period of flower opening, since the stigma was observed to desiccate before floral tube abscission. Changes in stigma colour and shape after pollen shed may be used as a basis for determining the onset and duration of stigma receptivity.

Kulkarni and Muniyamma (1998) evaluated changes in stigma morphology and, while these authors did not directly measure stigma receptivity, reported that the presence of a shiny sugary drop on the stigmatic surface is likely to represent stigma receptivity. It was further reported that greatest proportion of the stigma with this morphological feature was consistently observed on the day after flower opening (Kulkarni and Muniyamma 1998). No observations of any stigma exudate were observed in the *S. album* accessions used in this study. However, further investigation of stigma receptivity and morphological changes may lead to visual associations between stigma receptivity and flower development stages, that could be employed in controlled pollination procedures in *S. album*. Stigma receptivity in both *S. spicatum* and *S. album* were reported to commence after the flower opens and attaining a peak 2-3 days later (Rugkhla *et al.* 1997). They further reported that pollen tubes grow more slowly in green compared with red, where they took 2 and 1 days respectively to reach the ovary.

Differences in the rate of flower development in *S. album* is most likely influenced by variation in environmental factors such as temperature, but further investigation is required to further examine its effects. Given the brief 'life' of the flowers in *S. lanceolatum* and *S. austrocaledonicum* the phenological variation was proportionally much greater than for *S. album*, such that the life of a flower in *S. lanceolatum* could vary by as much as 100% (i.e. 12 to 24 hours). In both species no visual changes in stigma morphology were detected. The timing and duration of stigma receptivity requires further investigation, but it is likely that these species are either slightly

protandrous (pollen shed before stigma receptivity) or pollen shed and receptivity occur simultaneously. This is proposed since pollen shed, particularly for *S. lanceolatum*, occurs throughout the period where the tepals are open and the stigma is available for pollination. Furthermore the upper part of the stigma is abscised concurrently with the floral tube, so the stigma is only available for pollination by 'large' insects during the opening of the tepals. In *Santalum* species, the most common pollinators are bees, flies, beetles, ants, butterflies and wasps (Jyothi *et al.* 1991; Kulkarni and Muniyamma 1998). Both ants and flies were commonly observed on the flowers of the three species in this study. Ants were often found to chew and remove the style at its base, although the purpose for this behaviour was not determined. From observations in this study it could be possible that small insects (such as thrips and ants) could penetrate the small openings in the tepals of *S. lanceolatum* and *S. austrocaledonicum* after the flower has closed. The frequency of such events and their influence on effecting pollination is not yet known.

5.2 Unpollinated flowers of *S. lanceolatum*

In this study, no fruit or seeds were set following isolation of flowers and restricting pollination of *S. lanceolatum*. This result suggests that this species does not possess a capacity for the development of parthenocarpic fruit or clonal seed. This result is similar to that found in *S. album* in China, where no seeds were found in flowers isolated from open pollination by bags (Ma *et al.* 2006).

5.3 Self-incompatibility in *S. lanceolatum*

Resolving the nature of the breeding system in *Santalum lanceolatum* is important for planning appropriate breeding strategies for its domestication. Such knowledge is also important in interpreting the nature and extent of genetic variability in natural populations of the species, and in turn it would lead to greater efficiency in the evaluation and use of this natural variation for plant breeding. The mean seed set per pollinated flower in *S. lanceolatum* was significantly greater following cross- compared with self-pollination, where seed set from cross-pollination was 5.8-times, greater than from 'selfing'. This result indicates a possible self-incompatibility mechanism(s) operating in this species. Rugkhla *et al.* (1997) proposed that both pre- and post- fertilisation self-incompatibility mechanisms were operating in *S. album* and *S. spicatum*. This study however, found that putative self-incompatibility mechanism(s) in *S. lanceolatum*, may either be incomplete, or subject to genetic variation between accessions, given that seed set, was affected following self pollination in 20% of genotypes tested. Furthermore, two self-pollination derived seeds were successfully germinated and have continued to grow for a period of 2 years without indication of any deleterious effects of inbreeding. Warburton (2000)

found that little to no sexual reproduction in natural populations of *S. lanceolatum* in Victoria due to pollen sterility in one and self incompatibility or pistil dysfunction in other populations. The populations in this study were found to consist of many ramets (derived from root suckers) of one clone, resulting from historical commercial exploitation. This combined with the findings of this study give greater weight to the possibility that self-incompatibility mechanisms operate in *S. lanceolatum*, but genetic variation in its expression exists within its natural populations. It is possible that any self-compatible genotypes present in the Victorian populations may have been removed during the period of uncontrolled harvesting.

These results are similar to those found by Muir *et al*. (2007) for *S. spicatum*, where one family showed a high level of inbreeding, which was contradictory to the high mean outcrossing rate (95.2%). These authors proposed that flowering of this family was non-synchronous with many other families, resulting in higher inbreeding. This flexibility in breeding strategy would be of advantage in continental Australian species dispersing and colonizing many islands in south-east Asia and Pacific (Harbaugh and Baldwin 2007). In *Santalum album* Ma *et al.* (2006) reported 24% of flowers with geitonogamous (same plant and different flower) self-pollinated set seed.

In this study all cross types (self-, intraspecific and interspecific) were carried out on a given individual ramet. Therefore it is possible that the reduced selfing rate recorded in this study could be due to competitive interactions between flowers with 'outcross' and those with 'self' pollen and preferential maternal resource allocation to those most competitive. It would be of interest to evaluate the percentage seed set between these three cross types, where each type is restricted to an individual ramet of a given genotype. This would remove any interaction effects that may have been operating in the present study.

5.4 Intraspecific hybridisation in *S. lanceolatum*

The mean level of seed set amongst crosses of eight genotypes of *S. lanceolatum* was 7.5% of pollinated flowers. Fruit set (and thus seed set, given a fruit is generally single seeded) from open pollinated *S. album* trees was less than 2-3% in China (Ma *et al.* 2006) and 5.2% in India (Sindhu Veerendra and Anantha Padmanabha 1996). Rugkhla *et al*. (1997) reported a final fruit set of 1.3% in controlled intraspecific outcross pollination of *S. spicatum* in Western Australia. These authors also found a 10% fruit set in controlled outcrosses of *S. album*, which was similar to the 9.4% found by Kulkarni and Muniyamma (1998) in India. While Ma *et al.* (2006) found that 2-3% of open pollinated *S. album* flowers set seed, this was increased to 14% during artificial outcross pollinations. These results suggest that while improved seed

set may be achieved using controlled pollination, several *Santalum* species produce an abundance of flowers but less than 10% of these typically develop into viable seed.

The significantly greater (i) number of seeds set per intraspecific outcross and (ii) percentage of unique intraspecific pollinations (genotype combinations) developing seed compared with self-pollinated flowers suggests a putative self-incompatibility mechanism. However the low germination rate (40%) for intraspecific outcross derived seed resulted in no significant difference in the number of seedlings between intraspecific and self-pollinated flowers. Further replication of this work is likely to reveal the exact nature of the low germination rate among 'intraspecific seeds'.

5.5 Interspecific crosses between *S. lanceolatum* and each of *S. album* and *S. austrocaledonicum*

Despite total geographic isolation and significant morphological divergence between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum* no reproductive barrier appears to exist between them because of equivalent or greater seedling production relative to the *S. lanceolatum* intraspecific cross. Seed producing two seedlings were found in crosses between *S. album* (♂) and *S. lanceolatum* (♀) was found, and although this is not unusual, the level (7.5% of seed) was elevated compared with all other crosses in this study and with *S. album* intraspecific crosses in controlled crosses in China where the frequency was (2.5%) (Ma *et al.* 2006).

It appears that *S. lanceolatum* has a particularly high cross compatibility with *S. austrocaledonicum*, but this result may be confounded by the use of only a single *S. austrocaledonicum* genotype (T1). It is possible that T1 may have a high general combining ability for cross compatibility with *S. lanceolatum* and therefore greater numbers of genotypes would need to be used in crosses to determine if the results in this study accurately reflect the cross-compatibility between these two species.

These results however, reflect similar findings with putative hybridisations between *S. yasi* and *S. album* in Fiji, with no apparent reproductive barrier or hybrid breakdown (Bulai and Nataniela 2005; Doran *et al.* 2005). Bulai (2007) further reported that spontaneous hybrids between *S. yasi* and *S. album* are now being produced in clonal seed orchards, and these hybrids appear to have higher vigour, wider environmental tolerances and are less dependant on forming host associations. Rugkhla *et al*. (1997) found that no seeds developed after 1930 reciprocal controlled pollinations between *S. spicatum* and *S. album*, and reported that strong incompatibility mechanisms operated between pollen and style, and possibly in the developing zygote.

Doran and Brophy (2005) proposed that interspecific hybrids may provide the opportunity to improve the planted form of sandalwood particularly given the good vigour of F1 hybrids between *S. album* and *S. yasi* observed in Fiji. Hybridisation between *S. lanceolatum* and *S. album* may be used to incorporate important characters from each of these species into a cultivar for use in commercial plantations. Combining characters such as straight form and fire tolerance from *S. lanceolatum* and high heartwood oil concentration and quality (%α- and β-santalol) from *S. album* in a cultivar may be possible provided additive genetic effects predominate in the characters of interest (general combining ability). However a more involved procedure of reciprocal recurrent selection would be necessary to combine the desirable traits from both species in cultivars if non-additive gene effects predominated in the F_1 hybrids (specific combining ability) (Eldridge *et al.* 1993).

Barriers to successful introgression were found to exist between *Eucalyptus crebra* and *E. melanophloia*, where Drake (1981) found the hybrid population produced only 10% of the capsule yield of either parental species which, under natural selection, would put the hybrids at a competitive disadvantage. While segregating populations can be generated through artificial hybridisation of *Chamelaucium uncinatum* with each of *C. megalopetalum*, *Verticordia plumosa* and *V. grandis*, the resulting progeny of all crosses were infertile (Growns *et al.* 2002) and therefore it was not feasible to carry out further breeding. The high level of cross-compatibility between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum* indicates the likelihood that they are not widely divergent genetically and chromosomally (few chromosome structural differences) and thus the transfer of characters, even those under quantitative genetic control, would appear to be feasible from interspecific crosses. While the high cross-compatibility between these three species indicates the likelihood that they are not widely divergent genetically, it would be necessary to evaluate the fertility and seed production level of both their F_1 hybrid and F_2 progeny, because it is possible that genetic divergence between the two species may not be significantly manifest until these post-hybridisation stages.

The apparent lack of interspecific barriers between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum* also has implications for the conservation of their natural stands. Given its low relative value it is unlikely that *S. lanceolatum* would be introduced into areas of natural populations of *S. album* or *S. austrocaledonicum*. Commercial plantings of *S. album* have however, already been established in some areas of Queensland with existing natural populations of *S. lanceolatum*. It is very much possible that gene flow will occur between the *S. album* plantings and the *S. lanceolatum* populations. It is unclear, whether such hybrid progeny would have an advantage in these environments and persist beyond 1 or 2 generations. These considerations however may need to be evaluated by those responsible for (a)

management of *S. lanceolatum* wild stands and (b) improvement of *S. album* germplasm for commercial production.

6 References

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