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RESEARCH PAPER

Patterns of hybrid seed production in adjacent seed orchards of *Acacia auriculiformis* and *A. mangium* in Vietnam

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Abstract

• ***Key message*** **Hybrid seed production in adjacent seed orchards of Acacia auriculiformis and Acacia mangium was influenced by (i) flowering times of the two species and (ii) the distances of parent trees from the inter-orchard boundary. Approximately 80% of hybridisation events were found within 60 m from the boundary.**
• ***Context*** Understanding pollen dispersal has important implications for breeding and seed production of pure species and hybrid *Acacia*.
• ***Aims*** We examined patterns of hybrid production in adjacent clonal seed orchards of *A. auriculiformis* and *A. mangium* in Vietnam.
• ***Methods*** We assessed the frequency of hybrid offspring using four species-diagnostic SSR markers in seed collected from a total of 72 trees (75 seedlings per tree) at distances ranging from 4 to 144 m from the inter-orchard boundary. The number of hybrid was determined from SSR allele peak sizes in pooled sample (10 seedlings per pool, 540 pools). Calibrations were developed from pools with known proportions of pure-species and hybrid material.
• ***Results*** Hybrid frequency differed significantly among individual clones ($P < 0.001$) but not between species (*A. mangium* = 3.4%, *A. auriculiformis* = 2.8%). Two late-flowering clones of *A. auriculiformis* yielded no hybrids. The level of interspecific hybridisation declined significantly ($P < 0.001$) with increasing distance, and no hybrid seed was produced by trees located more than 116 m from the inter-orchard boundary.
• ***Conclusion*** Pooling of tissue samples for analysis of species-specific DNA polymorphisms was an efficient, low-cost strategy for detecting hybrid genotypes among the offspring of pure-species parents, and a low rate of associated error was demonstrated. The inferred decline in inter-species pollen flow with increasing distance from the boundary between the two orchards provides guidance for the design of hybridising orchards and isolation requirements to prevent contamination of seed orchards by external pollen sources.

Keywords Clonal seed orchard · Contamination · Hybrid seed · Pollen dispersal · Seed orchard design and management · Seed production

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1 Introduction

Acacia auriculiformis A. Cunn. Ex Benth. and *Acacia mangium* Willd., which are native to northern Australia, New Guinea and the Papua Province in Indonesia, were introduced to Vietnam in the 1960s. Together with their interspecific hybrid (hereafter called acacia hybrid), they have become the most important forest plantation species in Vietnam, being grown primarily on rotations of 5–8 years for the production of pulpwood and small sawlogs (Nambiar and Harwood 2014). Breeding programs for these two tropical species have been implemented in Vietnam since the mid-1990s (Hai et al. 2015; Hai et al. 2008; Harwood et al. 2015). Seed production areas, seedling seed orchards and clonal seed orchards (CSOs) have been established for the production of improved seed.

Hybridisation between *A. auriculiformis* and *A. mangium* has been recorded in natural forests and plantations (Kha 2001; Sedgley et al. 1992a), and selected acacia hybrid clones have become important for wood production in Vietnam because of their high growth rate and wide adaptability to various environments. Vietnam's acacia hybrid plantation area exceeded 0.5M ha in 2014 (Nambiar et al. 2015) but is based on only about 10 production clones (Kha et al. 2012). Development of a broader base of acacia hybrid clones is necessary to enhance the productivity and biosafety of this large plantation estate.

Acacia hybrid genotypes can be produced via controlled pollination. However, because acacia flowers are small and difficult to emasculate, this requires much time and expense (Nghiem et al. 2016). Accordingly, accessing new acacia hybrid genotypes has relied mainly on detection of hybrid individuals within open-pollinated progenies of the parental species. Hybrid seed production is most likely in situations where adjacent trees of the two species flower synchronously (Wickneswari and Norwati 1992). However, the effect of distance and genotype on hybrid seed production of these taxa in the Vietnamese environment is not well understood.

Pollen contamination from unimproved populations and other taxa can decrease the expected genetic gains from breeding (White et al. 2007). In pure-species breeding populations and seed orchards of *A. auriculiformis* and *A. mangium*, it is desirable to minimise contamination from nearby routine *Acacia* plantings. It is therefore important to understand patterns of pollen movement, both for design of plantings for hybrid seed production and isolation of pure-species seed orchards from contamination.

As part of Vietnam's acacia breeding programs, paired clonal seed orchards of *A. mangium* and *A. auriculiformis* were established in 2001 in southern Vietnam (Harwood et al. 2015; Le et al. 2017) to produce both pure-species and acacia hybrid seed. Putative hybrids have usually been identified by morphology assessment at the nursery stage (Gan and Liang 1992) but this has been shown to result in an over-estimation of hybrid occurrence (Gan and Liang 1992; Le et al. 2017). Recently, species-diagnostic markers developed by Le et al. (2016) provide a more accurate method to identify hybrids or check the status of putative hybrid individuals (Le et al. 2017).

Acacia auriculiformis and *A. mangium* are insect pollinated and preferentially outcrossing (Butcher et al. 2004; Moran et al. 1989; Sedgley et al. 1992b). Pollinator behaviour thus contributes to the pattern of pollen dispersal. In their natural environment, bees of the genus *Trigona* are the most numerous insect visitors to *A. mangium* flowers and had the highest numbers of acacia polyads (composite pollen grains) on their bodies (Sedgley et al. 1992a, b). Honeybees were observed to be the main pollinators of *A. auriculiformis* and *A. mangium* in Vietnam when they were planted together in a hybridising orchard (Nghiem et al. 2011). For bee-pollinated plants, cross-pollination is mostly over short distances (less than 40 m), although the nature of pollinator behaviour means that occasional polyads may travel much further (up to 3.2 km) (Burczyk et al. 2004; Dick et al. 2003; Jha and Dick 2010).

Pollen dispersal parameters can be estimated through paternity analysis using genetic markers. Using 12 microsatellite markers to assess pollen dispersal in two seedling seed orchards of *A. mangium* in Indonesia, Yuskianati and Isoda (2013) concluded that approximately 80% of all crosses were between trees separated by 40 m or less. In a parentage analysis of an *A. mangium* seed orchard in Indonesia, Nurtjahjaningsih (2016) found that pollinations occurred over distances ranging between 15 and 150 m. Longer distances of pollen movement have been recorded in some *Acacia* species, but these species have extrafloral nectaries and may be bird pollinated so patterns may be quite different to those of tropical *Acacia*. For example, maximum pollinator dispersal distances exceeded 1870 m in *A. woodmaniorum* Maslin and Buscumb, a species that occurs in small, isolated populations in dryland Western Australia (Millar et al. 2014), and occasional hybridisation between native populations of *Acacia saligna subsp. saligna* and subsp. *lindleyi* (Labill.) H.L. Wendl. was detected over distances of around 1600 m (Millar et al. 2008; Millar et al. 2014).

The aim of this study was to investigate patterns of hybrid seed production in adjacent CSOs of *A. auriculiformis* and *A. mangium* in southern Vietnam. Factors likely to influence the production of hybrid seed, including species and genotype effects, flowering phenology and distance of individual trees from the inter-orchard boundary were examined. Hybridisation rates were estimated using species-diagnostic SSR markers. Practical applications of the finding to hybrid seed production and seed orchard management are considered.

2 Materials and methods

2.1 Plant material

Adjacent clonal seed orchards (CSOs) of *A. auriculiformis* and *A. mangium* were planted in 2001 at Bau Bang (south Vietnam), for the production of both pure-species and hybrid seed. The orchards, both rectangular in their layout, shared a common boundary, which we refer to as the inter-orchard boundary. They were surrounded by plantations of cashew nut and rubber for approximately 100 m and *Acacia* beyond that. The orchards included 120 clones of *A. auriculiformis* and 100 clones of *A. mangium*, with each orchard having 8 replicates of 2-tree plots of each clone and initial spacing of 4 m × 2 m between trees. Thinning, conducted in 2006 to promote canopy development and seed production, removed one tree in each two-tree plot and completely removed 20 clones of *A. auriculiformis*.

Open-pollinated seedlots were collected from the orchards in 2009. Layout of the orchards at the time of seed collection is shown in ESM 3. Seed was stored at the Institute of Forest Tree Improvement and Biotechnology (Vietnamese Academy of Forest Sciences—VAFS). From the seed still available in 2016, 72 individual-tree seedlots were selected for the study. Seventeen clones of *A. auriculiformis* were represented by seedlots from either two or three ramets per clone and 18 clones of *A. mangium* by two ramets per clone (Table 1, ESM). For each clone, one ramet close to the inter-orchard boundary and one ramet as far as possible from the boundary was selected. Clones 14 and 1f of *A. auriculiformis* were both represented by 3 ramets. Clones in these seed orchards had previously been fingerprinted using SSR markers (Le et al. 2017), and most were found to be true to their species, but four clones in each orchard were found to be hybrids (either F_1 or backcross). Locations of the 16 hybrid trees remaining at the time of seed collection are shown in Fig. 1 in ESM 3. We avoided sampling trees within 10 m of these known hybrid individuals.

At least 80 seeds per seedlot were nicked at the distal end and germinated at 25 °C in petri dishes containing two layers of filter paper moistened with tap water. Germination was approximately 90%, and germinants were transplanted to plastic pots (2 plants of the same seedlot per pot) that contained a mix consisting of 7 parts composted fine pine bark and 4 parts coarse washed river sand and watered daily.

2.2 Phenology assessment

Flowering phenology was assessed in 2015–2016 to provide an estimate of the degree of flowering overlap between the two species. Layout of the *A. auriculiformis* CSO was

Table 1 Species contributions to the 13 pool types used to test the pooling strategy and accuracy of predictions of numbers of hybrid individuals in each pool type using SSR markers across 4 replicates. The species composition of the 13 pool types is shown as well as their expected mean peak ratio (*q* value, see "Materials and methods" for full explanation) and the *q* value range that was used to assign hybrid number. *A. auriculiformis* is abbreviated as AA, *A. mangium* as AM and AH as acacia hybrid (AM x AA)

Pool type	Ratio of species-specific alleles	Expected mean peak ratio for *A. mangium*–specific alleles	Peak ratio range (*q* value) for assigning no. of hybrid seedlings	Number of replications (pools)	Number of pools successfully predicted	No. of pools under-predicted	No. of pools over-predicted
10AA	20AA:0AM	0	0–0.004	4	4		
9AA:1AH	19AA:1AM	0.05	0.005–0.074	4	3		1
8AA:2AH	18AA:2AM	0.10	0.075–0.124	4	2		2
7AA:3AH	17AA:3AM	0.15	0.125–0.174	4	3		1
6AA:4AH	16 AA:4AM	0.20	0.175–0.224	4	2		2
5AA:5AH	15AA:5AM	0.25	0.225–0.274	4	2	1	1
10AH	10AA:10AM	0.50	0.475–0.524	4	3	1	
5AM:5AH	5AA:15AM	0.75	0.725–0.774	4			4
6AM:4AH	4AA:16AM	0.80	0.775–0.824	4	2	2	0
7AM:3AH	3AA:17AM	0.85	0.825–0.874	4	1		3
8AM:2AH	2AA:18AM	0.90	0.875–0.924	4	2		2
9AM:1AH	1AA:19AM	0.95	0.925–0.995	4	3		1
10AM	0AA:20AM	1	0.996–1.0	4	4		
Total				52	31	4	17

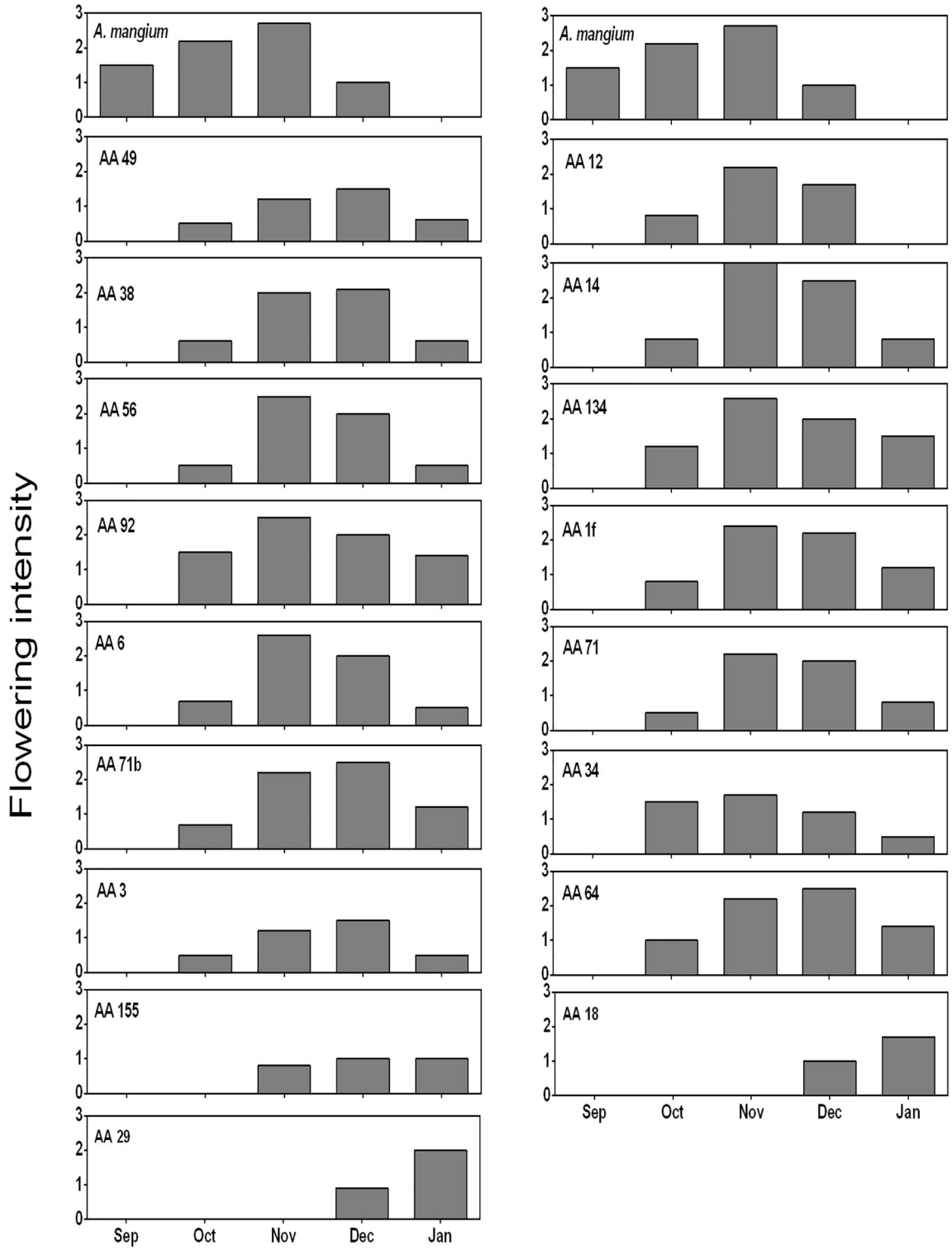

Fig. 1 Monthly average flowering intensity of *A. auriculiformis* (AA) clones in comparison with *A. mangium* (mean of 32 *A. mangium* trees shown at the top of both columns to facilitate comparison with each *A. auriculiformis* clone)

unchanged from 2009, and the number of remaining ramets per clone ranged from 5 to 8, enabling the phenology of the 17 clones selected for this study to be assessed. The *A. mangium* CSO orchard had been cut down in 2009, so we were unable to collect phenology data from the 18 studied *A. mangium* clones. However, progenies of most of the clones in the *A. mangium* CSO were planted on the former CSO site in a progeny trial planted in July 2009. Phenology data were

collected from progenies of 16 of the *A. mangium* CSO clones, assessing 2 sibs from each clone. Flowering time and flowering intensity of *A. mangium* and *A. auriculiformis* were recorded fortnightly from October 2015 to the end of January 2016. The flowering intensity of each tree was scored visually using the following categories: (0)—no flowering; light (1)—up to 1/3 of the crown bearing open flowers; moderate (2)—from 1/3 to 2/3 of the crown bearing open flowers; and heavy (3)—more than 2/3 of the crown bearing open flowers. To obtain the monthly flowering intensity of each clone, the scores of all ramets for each clone over each month were averaged. Differences in flowering phenology between the 16 *A. mangium* families were not evident, so a single monthly average flowering intensity for *A. mangium* was calculated from all 32 trees surveyed.

2.3 DNA isolation and pooling strategy

In order to reduce the labour and cost of genotyping, seedlings from the same seedlot were pooled. Each pool included equal quantities of phyllode material (approximately 10 mg) from 10 individual seedlings at the same development stage. Tissue was crushed to a powder in liquid nitrogen and the Qiagen protocol used for DNA extraction (www.qiagen.com/handbooks). DNA concentration and purity were assessed using gel electrophoresis and by comparison with Lambda *Hin*d III molecular weight standard. In total, DNA was isolated from 540 pools (5400 seedlings). The number of seedlings per seedlot was 75, tested as 7 pools of 10 seedlings with the five remaining seedlings combined with five seedlings derived from another ramet of the same clone. If hybrids were detected in the combined pool, supplementary runs of five seedlings from each maternal ramet were conducted.

2.4 PCR conditions and PCR product analysis

PCRs were conducted for all 540 pooled DNA samples using a GeneAmp PCR system 9700 (Applied Biosystems, USA) with a final volume of 12.5 μl, consisting of 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each forward and reverse primer, 0.5 U Taq DNA polymerase (Invitrogen, Massachusetts, USA) and 20 ng of genomic pooled DNA. The primers from the 6 species-diagnostic SSR markers recently developed (Le et al. 2016) were tagged with fluorescent dyes NED, 6-FAM, HEX and ROX on their forward primers and multiplexed. The loci were amplified using a series of touchdown programs with annealing temperatures (T_a) spanning 10 °C. The annealing temperature range was centred on the average optimal T_a for the six pairs of primers. The touchdown program initial annealing temperature was 60 °C, which decreased to 50 °C over 20 cycles, achieved by decreasing the temperature by 0.5 °C every cycle. This was followed by a further 20 cycles using a 50 °C annealing temperature. Cycling was composed of a 95 °C hold for 1 min, annealing temperature for 1 min and 72 °C for 1 min. Cycling was preceded by a hold at 95 °C for 5 min to provide a "hot start" and finished with a final hold of 10 min at 72 °C. PCR products were separated using an ABI 3730 DNA Analyzer (Applied Biosystems, USA) by the Australian Genome Research Facility (http://www.agrf.org.au). Raw data were analysed using GeneMapper 3.7 (ABI, USA) program, and peak sizes for each possible allele were recorded (see below for further explanation).

2.5 Statistical analysis

We used q value to predict the number of hybrid individuals in the pools. The q value was calculated as the mean ratio of diagnostic peak sizes that was obtained from four markers (e.g. q value = peak value of *A. mangium* alleles/(peak value of *A. mangium* alleles + peak value of *A. auriculiformis* alleles). Pools comprising 10 samples from either pure species were expected to yield no detectable peak representing alleles of the other species (q = 0 or 1). A pool was predicted to contain one hybrid if q was within the range to 0.005–0.074; if q = 0.075–0.124, the pool was predicted to have two hybrids, etc. (Table 1). When more than one diagnostic allele per locus was present for a species, their peak sizes were summed.

The total number of hybrids in each seedlot was estimated by summing the numbers of predicted hybrids across all pools of that seedlot. To investigate genetic and distance effects on the rate of hybridisation, a linear model was fitted with the number of hybrid individuals (out of 75 progenies tested) per tree as the dependent variable and species and clone within species as factors and distance and (distance × species) as covariates, using PROC GLIMMIX of SAS (Version 9.4, SAS Institute). A square root transformation of the dependent variable was used to improve the distribution of residuals. To find the curve which best described the decay in the number of hybrid with distance, a variety of non-linear models (negative exponential, power, logistic and logarithmic functions) were fitted using the SPSS program (https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software).

2.6 Test of pooling strategy

We tested the accuracy of our pooling technique to determine the frequency of hybrids between *A. auriculiformis* and *A. mangium*. We used 50 pure *A. auriculiformis* (AA), 50 pure *A. mangium* (AM) and 25 control-pollinated F_1 hybrid (AH) seedlings to construct 13 types of pools of 10 samples having various contributions of alleles from each species (Table 1). Because the frequency of hybrids was expected to be low, we

constructed pools dominated by either pure species and with a low frequency of hybrids (0 to 5 hybrids for each species). The pools were prepared by mixing 10 mg of phyllode tissue from each seedling and DNA from the pooled sample extracted using the Qiagen protocol as described above. Each pool type was replicated four times using different seedlings giving a total of 52 test pools.

Our pooling technique assumed that PCR would be quantitative and q values would increase additively with increasing hybrid representation in a pool. We tested this assumption separately for each SSR loci. We examined the accuracy of hybrid detection with the pooling strategy in two different ways. Firstly, we calculated linear regressions of observed q values in the test pools (based on peak ratios) versus expected q values (based on known composition of each of the test pools) using SPSS (https://www.ibm.com). Secondly, we compared the predicted versus expected number of hybrids for all 52 individual test pools.

3 Results

3.1 Flowering phenology

Acacia auriculiformis clones flowered later than *A. mangium* over the period from September 2015 to January 2016 (Fig. 1). *Acacia mangium* commenced flowering in September and was finished by December, whereas, *A. auriculiformis* flowered from October to January (Fig. 1). *Acacia auriculiformis* peak flowering was generally observed in November and December. One period of synchronous flowering with high flowering intensity (moderate to heavy) in both species was observed in November, with the exception of clones 155, 18 and 29 of *A. auriculiformis* which had little overlap in flowering with *A. mangium* in December.

3.2 Evaluation of hybrid prediction using pooling strategy

We evaluated separately each of the six species-diagnostic SSRs. Two markers (AH3_17, AH08) had regression results which were inconsistent with expectation of a linear increase in q value with increasing hybrid representation, with linear regression accounting for less than 60% of the variance in observed versus expected allele peak ratios (Table 1 in ESM 1), and these were dropped from further analysis. The other four markers showed much better relationships with linear regressions explaining 96–99% of the variance. However, out of a total of 52 pools, 17 were over-predicted and 4 under-predicted, by one hybrid individual in all cases (Table 1). Thus 21 out of 52 pools (40%) were in error by 10% (over- or under-estimate of 1 out of 10 seedlings), for a combined error rate of 4% in the evaluation population.

3.3 Frequency of hybrid progeny

Out of a total of 540 pools, 414, 101, 21, 3 and 1 pools were predicted to have 0, 1, 2, 3 and 4 hybrids respectively. No pools were predicted to have more than four hybrids. Of 5400 progenies, 156 were predicted to be hybrids. However, applying the error rates determined in the evaluation, the overall number of hybrids is likely to be slightly over-estimated, as discussed below.

Acacia mangium and *A. auriculiformis* yielded similar percentages of hybrid seedlings overall (3.4% and 2.8%, respectively). The difference in hybrid frequency between the species was not significant (Table 2). Clones differed significantly in their yields of hybrid seeds ($P < 0.001$; Table 2). Distance from the inter-orchard boundary was the most important factor influencing the production of hybrid seed yields ($P < 0.001$, Table 2). The species × distance interaction was not significant.

The highest frequency of interspecific hybridisation was close to the inter-orchard boundary, and frequency declined with increasing distance in both species (Fig. 2). Trees within 16 m of the inter-orchard boundary (16 trees/species) yielded on average 9.1% hybrid progeny. The average level of interspecific hybridisation for trees between 16 and 64 m of the boundary (24 trees per species) decreased to 4.5%. Trees between 68 and 112 m from the boundary (total of 24 trees) produced an average of 1.2% hybrids, and trees located more than 116 m from the inter-orchard boundary produced no hybrid seeds. Approximately 80% of hybrid individuals were from mother trees that were located within 52 m from the inter-orchard boundary. The overall decline in the hybridisation rate with distance was best modelled by a power function:

$$\text{Number of hybrid progeny} = -2.52 \ln(\text{distance}) + 11.65, R^2 = 0.67.$$

There were major departures from this curve, for example, one individual of *A. mangium* 44 m from the inter-orchard

Table 2 Univariate analysis of covariance of the number of interspecific hybrid individuals per tree (square root transformed) in adjacent clonal seed orchards of *A. auriculiformis* and *A. mangium*

Effect	Numerator DF	*F* value	Probability
Clone	33	3.14	< 0.001
Species	1	0.03	0.87
Distance	1	60.8	< 0.001
Distance × species	1	0.27	0.60

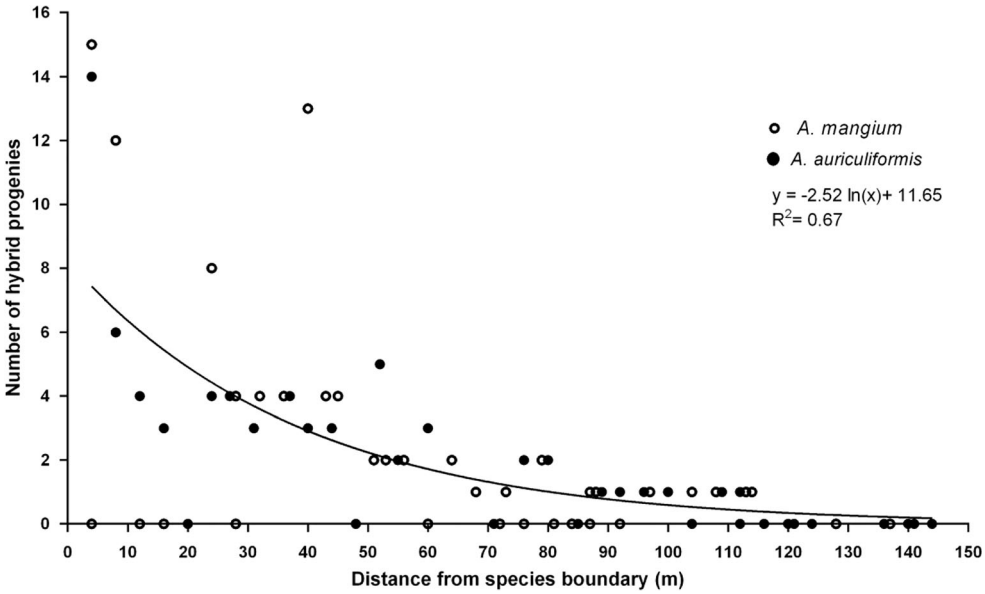

Fig. 2 Effect of distance from inter-orchard boundary on the number of interspecific hybrids detected in 75 progenies per tree for *A. auriculiformis* and *A. mangium* trees (each point in the graph is the result from one tree). The curve shows the power function providing the best fit to the data

boundary had 13 hybrid offspring among 75 progenies tested (Fig. 2).

Across both species, 27 out of 72 trees yielded no hybrids and some of these trees were close to the inter-orchard boundary. Three *A. auriculiformis* and five *A. mangium* clones yielded no hybrids from either of their two ramets (Table 2 in ESM 2). *Acacia auriculiformis* clones 18 and 29, which had only 1 month of overlap in flowering with *A. mangium* (Fig. 1), and clone 155, which had low flowering intensity and only 2 months of flowering overlap, produced no hybrid seeds.

4 Discussion

In this study, the number of hybrids in samples of open-pollinated seed of acacia trees was estimated from quantitative data on species-diagnostic allele frequencies obtained from pooled DNA samples of progenies from single trees. With this approach, a large number of seeds could be screened but the procedure was not error free. In the validation study, two of the six SSR loci failed to predict correctly the number of diagnostic alleles in pools and were dropped. We believe the most likely reason for the lower reliability of two of the markers was the changes to PCR conditions used in the present study compared with those in Le et al. (2016). In this earlier study, 16 markers were amplified in four different PCR mixes with four different annealing temperatures. In the present study, the six species-distinguishing markers that were PCRed together came from across the four different mixes in Le et al. (2016). We opted to use a touchdown PCR approach with a range of temperature which encompassed the annealing temperatures used in Le et al. (2016). Previous studies have shown that an important step in developing a PCR-based pooling strategy is the selection of loci (Skalski et al. 2006). Using the best SSR loci, the number of hybrids was still incorrectly estimated in approximately 40% of the pools and the majority of these were over-estimates. However, in all cases, this error was only by one out of ten individuals in the pool for a combined error rate of 4% in the evaluation population. This error rate is lower than when using seedling morphological characters to distinguish pure species from hybrids (*A. mangium* x *A. auriculiformis*) (Gan and Liang 1992; Le et al. 2017). In the main experiment, pools with no hybrids should all have been predicted correctly and pools with only one hybrid should not have been under-predicted. However, some of the pools predicted to have 2, 3 or 4 hybrids in the main study probably actually had 1, 2 or 3 hybrids respectively. Considering the over-estimation rates for the 2-, 3- and 4-hybrid pool categories detected in the validation study (Table 1), over-prediction of hybrid individuals in our main study may have been as high as 9%. However, the exact error rate in the main experiment cannot be predicted precisely. In future studies of this type, a strategy of sub-dividing pools for which 2 or more hybrids are predicted and re-running them as two separate pools of 5 seedlings could greatly reduce the associated errors and uncertainty.

The hybrid and backcross trees present within each species orchard may have produced pollen with hybrid-identifying alleles. We could not account for the contribution of these trees on the estimates of hybrid frequency. However, we consider it would be small. The hybrid and backcross trees represent only 2% of the trees in both orchards, and the contribution of species-diagnostic alleles from the backcross and F_1 hybrid

trees should be a quarter and half, respectively, of that of a pure-species tree. We avoided sampling trees within 10 m of known hybrid and backcross individuals in each orchard reducing their influence to some extent. We tested whether there was a correlation between the number of hybrids found in each tree across both orchards and their distance to the nearest hybrid or backcross tree and found this was not significant ($r = 0.13$, $P = 0.26$).

4.1 Influence of distance from inter-orchard boundary on hybrid seed yields

The frequency of hybrids declined with increasing distance between the species. This suggests that pollen flow of the two species decreased exponentially with increasing distance from the pollen source, with very little pollen travelling beyond 100 m. The highest rates of individual hybrid occurrence (15/75 progeny in *A. mangium* and 14/75 in *A. auriculiformis*) equate to 19–20% hybrid frequency. This agrees with results of an earlier study in Malaysia, in which trees of each species separated by approximately 10 m yielded 6.9% hybrids (range of 0.7 to 21.7%) for *A. mangium* and 9.3% (range of 2.9 to 14.7%) for *A. auriculiformis* (Wickneswari and Norwati 1992).

In this study, 80% of hybridisation events occurred within 52 m of the inter-orchard boundary (ESM 4). This was consistent with a study in pure–*A. mangium* seed orchards in Indonesia (Yuskianti and Isoda 2013) where 80% of cross-pollinations occurred between trees separated by 40 m or less, although occasional dispersal events took place over 100 m. Similar results were found in planted stands of *A. saligna* where the average pollen dispersal distance was 37 m with the majority of progeny sired by paternal trees within a 50-m neighbourhood of the maternal trees (Millar et al. 2008). Our results are also consistent with studies on pollen dispersal in *Eucalyptus* species, which are also mainly pollinated by bees, with almost all dispersal event occurring less than 300 m from pollen sources.

The floral characteristics of acacias are clearly adapted to biotic rather than wind pollination (see Sedgley and Griffin 1989), so the behaviour patterns of the pollen vectors affect the hybrid seed production in this study. Honey bees and native bees are the main pollinator of tropical acacias (Nghiem et al. 2011; Sedgley et al. 1992a). In Vietnam, farmers commonly place hives of honey bees in acacia plantations for honey production. The introduction of bee hives within seed orchards has been shown to change the natural pattern of seed productions, and the number and positioning of hives will affect pollen dispersal patterns (Moncur et al. 1995). In 2009, there were beehives within 200 m but not within the orchards that we studied. In Brazil, honey bees collect *Eucalyptus saligna* Sm. pollen of different plants in one visit and were found to promote cross-pollination up to 100 m, decreasing gradually up to a distance of 300 m (Pacheco et al. 1986).

4.2 Effects of flowering phenology and genetic differences

There were significant differences between clones within species in hybrid yields. The limited overlap in flowering time of some clones of *A. auriculiformis* with *A. mangium* is a likely contributing factor. Fourteen out of 17 *A. auriculiformis* clones had substantial flowering overlap with *A. mangium* (Fig. 1) and all yielded hybrid seeds. The three *A. auriculiformis* clones which produced no hybrid offspring commenced flowering later than the other *A. auriculiformis* clones and had only 1 or 2 months of flowering overlap with *A. mangium.*

There were five *A. mangium* clones that yielded no hybrid seeds. Unfortunately, phenological information on these *A. mangium* clones was not available. Early-flowering clones of *A. mangium* would have had little overlap with *A. auriculiformis*. The more overlap in flowering time, the more chances to find hybridisation in the orchard (Josue 1992). It has been suggested that flowering time is probably the major determinant of the levels of hybridisation between plantations of *Eucalyptus nitens* H Deane & Maiden and adjacent natural stands of *E. ovata* Labill. and *E. viminalis* Labill. Hybridisations of *E. nitens* with *E. ovata* have been observed, whereas no hybrids between *E. nitens* and *E. viminalis* were detected. This was attributed to the lack of overlap in flowering time between *E. nitens* and *E. viminalis* whereas *E. nitens* did overlap with *E. ovata* (Barbour et al. 2002). Our method did not allow full paternity analysis of the seeds we assayed (Burczyk et al. 2004) and assumed that all interspecific pollen originated from the inter-orchard boundary. In fact, some interspecific pollen probably came from trees of the other species beyond the edge trees along the inter-orchard boundary, further back within the respective seed orchards. This would result in the dispersion distances shown in Fig. 2 being somewhat under-estimated.

Phenology was studied in 2015 and 2016, and flowering phenology may have differed from that in the 2008 flowering season prior to seed collection in 2009. However, other studies of flowering in these *Acacia* species across multiple years have found similar phenology rankings of the two species and of individual clones over successive years. In a separate nearby smaller clonal hybridising orchard containing ten clones each of the two species, Nghiem et al. (2011) observed that the mean timing of peak flowering for *A. mangium* was about 2 months in advance of that of *A. auriculiformis* in both the 2008 and 2009 flowering seasons. While there was substantial overlap in species flowering periods both years, there would have been no opportunity for interspecific hybridization for early-flowering *A. mangium* or late-flowering *A. auriculiformis*. In a 3-year study of the flowering of *A. mangium* and *A. auriculiformis* clones in Okinawa (26° N), Kato et al. (2012) observed that *A. mangium* clones commenced flowering a month or two earlier in the season than did most *A. auriculiformis* clones. Initiation and duration of flowering differed significantly among the 23

A. auriculiformis clones, with clone rankings consistent over successive years. A broadly similar pattern of earlier flowering initiation in *A. mangium*, but some overlap with *A. auriculiformis*, was observed at Atheron, Queensland (latitude 17° S), but at Tawau, Malaysia (6° N), flowering of both species was sporadic. There were several minor peaks through the course of 2 years, some of these species peaks overlapping and others not (Sedgley et al. 1992a). It appears that seasonal trends in temperature, day length, sunlight and/or rainfall influence the flowering phenology of both species, so geographic location and the associated local climate will influence the extent of inter-species mating that can be achieved in hybridising orchards.

4.3 Implications for seed orchard design and management

The similar rate of hybrid production from the two species confirms the lack of crossing barriers noted in previous studies (Nghiem et al. 2013; Sedgley et al. 1992a, b). The hybrid yields would have been higher if more trees overlapped in flowering time. Flowering time has been found to be highly heritable in *Eucalyptus globulus* (Jones et al. 2011). If, as is apparent from the study of Kato et al. (2012), the same is true in these tropical *Acacia* species, it would be possible to select clones of both pure species to achieve greater overlap in their flowering time. However, this would reduce diversity in the breeding populations for hybrid generation. Considering planting designs to promote hybridisation, alternating single rows of *A. auriculiformis* and *A. mangium*, at sufficiently wide spacing to promote canopy development of both species, would seem likely to maximise the proportion of hybrid seed produced. Use of a wide range of genotypes seems advisable, rather than using a limited set of genotypes of *A. auriculiformis* and *A. mangium*, some of which might not overlap in their flowering times. By monitoring flowering time in such seed orchards, the genotypes that overlap the most in flowering time can be identified. Seed collection and hybrid detection can target those trees and maximise the chance of identifying hybrids. Placing beehives in seed orchards when flowering of the two species overlaps is also recommended to promote pollen movement between the species (Moncur et al. 1995). Controlled pollination using stored pollen of early-flowering *A. mangium* genotypes to late-flowering *A. auriculiformis* genotypes is also an option for hybrid production, although technically demanding and expensive.

The acceptable level of external contamination in seed orchards and breeding populations depends on the purpose of seed production. For production orchards of *A. mangium* and *A. auriculiformis*, levels of about 3%, the orchard averages detected in the current study, would be acceptable as such levels would not lower genetic gain and hence plantation productivity appreciably, even if the seedlings derived from the external pollen source performed poorly (White et al. 2007, Ch. 16). We studied two orchards, each approximately square in their layout and slightly over 1 ha in area and each with a potential contamination source contiguous to one of their four borders. Mean levels of contamination would have been higher if they had each been surrounded by potential sources of contamination on all four of their borders. Orchard size is another determining factor of contamination levels since, other things being equal, increasing orchard size above 4 ha should yield an increasing proportion of orchard trees further than 100 m from the nearest orchard boundary, which on the basis of our study should receive very low levels of contamination. Contamination is a more critical issue for breeding orchards than for production orchards, since the default assumption for accurate estimates of genetic parameters and accurate selection of individuals for further breeding is that no offspring from the breeding orchard will have external pollen parents. The levels of contamination approaching 10% observed in trees close to the inter-orchard edge in this study would be unacceptably high for breeding purposes. Around some orchards in Vietnam, buffer rows of *Eucalyptus* have been planted to reduce pollen contamination from external pollen sources. The effectiveness of this measure has not yet been quantified. In the system and year under study, a separation of 116 m was enough to prevent any hybridisation from an interspecific contaminating source. The flowering phenology of the orchard genotypes and potential contaminants will influence contamination—pollen transfer from synchronously flowering trees of the same species would probably be higher than what was found in the current study. The separation distance to prevent contamination will also vary depending on factors such as bee management and pollen source/sink intensity (Dick et al. 2003; Richards 1997). Nonetheless, our results strongly suggest that an isolation distance of 100 m from nearby potential pollen parents would greatly reduce contamination of *A. auriculiformis* and *A. mangium* orchards.

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Data availability Raw measurement relating to this study are available in the University of Tasmania Open Access Repository (Le et al. 2019). Le S, Harwood CE, Nghiem CQ, Griffin AR, Vaillancourt RE (2019) Number of hybrid per clone and ramet, Son Le et al. 2019. Version 05 March 2019. University of Tasmania. [Dataset]. https://eprints.utas.edu.au/29111/.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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