Hort Innovation

Final Report

Macadamia Second Generation Breeding and Conservation

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Summary

Macadamia (*Macadamia integrifolia, M. tetraphylla* and their hybrids) is native to the subtropical rainforests of south-eastern Queensland and north-eastern New South Wales. The Australian macadamia industry has a farm gate value at over $250M, with around 45,000 tonnes of nut-in-shell produced yearly. The number of hectares under planting and number of Australian growers is increasing, and the need for genetically improved varieties is vital. This breeding project uses multiple strategies and techniques to breed new varieties for a more profitable Australian industry.

Continuous and on-going consultation with key industry stakeholders has informed the objectives and research paths in the breeding program. Priority traits essential for growers include high nut-in-shell yield per tree, small tree size, high kernel recovery (percentage of kernel to nut-in-shell; KR), precocious trees producing early fruit, resistance to husk spot and other pests and diseases. Themain topics investigated throughout the current breeding project were: creation of second generation of progeny, evaluation of existing progeny,improvement efficiency of breeding, use of DNA technology, rootstock trials for increased productivity, economic analysis and extension to growers, pathology research, and wild germplasm trials.

Increasing the number of progeny was a key project objective and this was accomplished with the field planting of progeny trials of over 10,000 trees (over 8,000 progeny plus standards and elites). Trials were planted at the Qld Government's Bundaberg Research Facility but the majority (6,117) were planted at grower properties at Childers, Alloway, Emerald and Pine Creek. Planting at grower properties has the benefit of increased grower involvement, wider sampling of production environments and reduced project costs. Most progeny were second generation hybrids resulting from the use of high performing elites selected from previous breeding.

Five new selections were made in 2019 from our progeny trials at the Bundaberg Research Facility. Details of their early yield performance are presented in this report. They are currently being propagated for inclusion in the fourth regional variety trial (MC17006). A further 18 elite accessions were selected from a high density trial at Nambour using a selection index that emphasized precocity, kernel recovery, tree size, nut-in-shell yield. These trees are being propagated for secondary evaluation at grower trial sites prior to inclusion in future regional variety trials. Details of the primary evaluation of these selections is provided in this report.

Selection strategies using yield component traits and genomics were explored in a PhD project to reduce the lengthy and laborious selection cycle involved in breeding. None of the studied yield component traits were effective for indirectly selecting for high yield. Genetic markers associated with nut characteristics and trunk circumference were identified, and a genomic model was developed to predict yield and yield stability. A new progeny trial of 1,600 trees was planted at Maroochy Research Facility which will employ this new genomic selection model in the MC19000 breeding project. The trees are planted at 4m x 0.5m making it one of the highest density plantings of a fruiting orchard recorded.

Wild material is not often used in breeding programs due to poor agronomic traits. However, wild germplasm is a potential resource for improving crops in the future through pest and disease resistance traits, ability to adapt to environmental change or potential to carry novel genes to improve fruit quality. To evaluate the potential of wild germplasm for breeding, the genetic diversity and population structure of macadamia species need to be determined. Genetic analysis determined that 287 of the genotyped trees were pure accessions, whilst 17 were admixtures of two or more species.

Keywords - 5-10 words

Yield, perennial, genetic improvement, tree, progeny, population, pollination, molecular markers, rootstock, economic analysis, genomics, pathology, wild germplasm

List of abbreviations

Abbreviation	Description
BALLO	Breeding trial planted at Alloway
BAMAM	Breeding trial planted at Amamoor
BBAFF	Breeding trial planted at Baffle Creek
BCHIL	Breeding trial planted at Childers
BDW	Breeding progeny dwarf
BEGYM	Breeding trial planted at East Gympie
BEMER	Breeding trial planted at Emerald
BHI	Breeding progeny with high index value
BHINK	Breeding trial planted at Hinkler Park
BHY	Breeding progeny with high yield efficiency
BNAMB	Breeding trial planted at Nambour
BNAMB18	Breeding trial planted at Nambour in 2012
BPINE	Breeding trial planted at Pine Creek
BQBRS	Breeding trial planted at Bundaberg Research Station
BTFRS	Breeding trial planted at Tropical Fdruit Research Station, Alstonville
CNM	cumulative nut mass
CW or CWA	Canopy weidth along the row
Cwa or CDA	Canopy depth across the row
DArT	Diversity Array Technology
ENF	Estimated number of florets per raceme
FSN	Flowers that set nuts (NPR / ENF)
gCNM	clonal value for nut in shell yield
gHG	clonal value for height of the tree
gTKR	clonal value for total kernel recovery
gVOL	Clonal value of the volume of the tree
gYE	clonal value for yield efficiency
H	Broad sense heritability
h2	Narroe sense heritability
HAES	Hawaiian Agricultural Experimentation Station
HGT pr THT	Total height
HGT_sk	Skirt height
IMCDW	Ian McConachie dwarf
IRR	Internal Rate of return
KWT or KW	Kernel weight
MIV	Macadamia industry variety
MIVIC	Macadamia Industry Varietal Improvement Committee
NCF	Annual Net Cash Flow
NIS	Nut-in-shell yield
NPR	Number of nuts per rachis
NPV	Net Present Value
NSD	Nut-in-shell diameter
NST	Shell thickness
NSW	New South Wales
NWT or NW	Nut-in-shell weight
PD	Nut pedicel diameter
QLD	Queensland
RDN	Rachis diameter at nut set
RHT	Hieght of the rootstock
RL	Raceme length
RSN	Racemes surviving from flowering to nut set
RTC	Rootstock trunk circumference
RVT	Regional Variety Trial
SHT	Scion height
STC	Scion trunk circumference
SWT	Shell weight
TC	Trunk circumference
TKR or KR	Total kernel recover
TNM	Total nus mass in the year
UQM	The University of Queensland Macadamia
vA	Additive genetic variance
vD	Dominant genetic variance
VOL	Volume of the tree
vR	Residual variance
WK	Percent Whole kernels

Introduction

Macadamia (*Macadamia integrifolia, M. tetraphylla* and their hybrids) is native to the subtropical rainforests of south-eastern Queensland and north-eastern New South Wales. The Australian macadamia industry has a farm gate value at over $250M, with around 45,000 tonnes of nut-in-shell produced yearly. The number of hectares under planting and number of Australian growers is increasing, and the need for genetically improved varieties is vital.

Industry funded macadamia breeding in Australia commenced with CSIRO field planting a population of 1,611 seedlings at Alstonville and Bundaberg in 1997-1998. This was coded as the B1.1 population; the first breeding population. Twenty elite accessions were selected from the B1.1 population and planted into regional variety trials (RVT3) from which four cultivars were released to industry in 2017 (MC11001 final report).

A second round of first generation progeny (coded B1.2) were planted by CSIRO in 14 trials at nine locations in NSW and Qld from 2001-2003. These 2,018 progeny were evaluated by QAAFI, CSIRO, DAF and DPI. In 2014, as part of the Hort Innovation project MC09021, industry representatives participated in a workshop which resulted in the selection of 23 elite accessions. These accessions are being propagated in 2019 for inclusion in MC17006, "Regional Variety Trial – series 4 led by DAF.

Testing of elite selections from the industry funded breeding program occurred concurrently with RVT3 through the Hort Innovation project MC09017 "Supplementary Grower Trial of Elite Macadamia Selections". This was conducted by the service provider AS & FC Bogg and managed by Mr Lindsay Bryen. In this project 12 elite accessions from the B1.1 and B1.2 breeding populations and the cultivar 'MCT1' were propagated at DeCortes in Qld and compared to standard cultivars 'HAES816' and 'A16'. This project has been funded since 2018 through the RVT4 project MC17006.

The number of progeny evaluated in the breeding program from its start in 1997 to 2008, a period of 21 years, was 3,629. An imperative of the breeding projects MC09021 and the current project MC14000 has been to increase the number of progeny. In the 5-years of MC0921 a further 3,555 progeny seedlings were planted in field trials at Nambour and Bundaberg. Details of the contribution of MC14000 follow in this report.

Breeding new varieties of horticulture tree crops is a lengthy and laborious process due to large tree size and long juvenile period. In commercial orchards, macadamia trees grow to a height of 12-15 m and may take 15 years to attain peak production. The large size and slow maturity of the trees pose problems for breeding, orchard management and farm profitability. Identifying scion/rootstock genetic resources for increased precocity and reduced vigour will significantly contribute to increase farm profitability and accelerate macadamia improvement program through reducing breeding cycle.

The objectives of the current breeding project MC14000 were to create new seedling populations and reduce the cost of breeding using multiple breeding strategies, introducing genomics to reduce breeding cycle, exploiting wild germplasm to increase genetic diversity and generating/evaluating rootstocks for high performance.

Methodology

1. Second generation of progeny

We have used two pollination methods to develop three distinct seedling populations during MC14000. A total of 8,828 progeny were produced from our second generation of breeding.

1.1 Populations produced by hand pollination (see Appendix 1)

A total of 2,711 progeny were developed by hand pollination. Progeny were derived from 151 families, which includes 34 parents. Progeny field trials were planted at the Queensland Bundaberg Research Station (QBRS) from 2012 to 2016. These trials consist of controlled cross-pollinated seedlings, buffer trees and clonally propagated parent and industry standard cultivars. The progeny are second generation breeding material from which we aim to select elite performing genotypes that will provide the next step of genetic gain for industry. Screening methods were those in MC09021 for the B1.2 population, and involved harvesting and weighing all nuts from each tree from years 3 to 7, as well as measurement in year 6 of nut drop pattern, kernel recovery and tree size. Traits are combined in a selection index to allow selection of elites. Elite-performing trees will have been measured for 25 more traits (described in MC09021) to allow culling on the basis of inferior horticultural characteristics. Final evaluation will be performed in 2024. Five elites were selected in 2019 from BQBRS12 and BQBRS13 trials based on early performance data. Plants were cinctured and grafted onto standard rootstock (H2) for planting in RVT-4 via the rolling front strategy that we have developed in MC17006.

1.2 Large populations at reduced cost (see Appendix 1.2)

In order to reduce costs of maintaining and evaluating progeny field trials and to allow production of larger populations, we introduced a polycross progeny breeding strategy. We developed three populations of progeny seedlings. These are described as follows:

1.2.1 B2.2 precocity trial (see Appendix 1.2.1)

For the B2.2_precocity trial, we developed 704 progeny from 39 families. Open pollinated seed were collected from precocious commercial cultivars, B1.1 elites, potential dwarf progeny and two accessions of *wild Macadamia jansen*ii. Seeds were germinated in 2009 and planted in 2011 at Maroochy research facility, Nambour. Progeny were evaluated for early growth, flowering, yield and nut characteristics. Selection was made at age 5 in 2016 using a selection index, described in appendix 1.2.1.

1.2.2 B2.2 polycross progeny planted at grower properties (see Appendix 1.2.2)

For B2.2, we planted 3,796 progeny from 11 half-sib families. Open pollinated seed from 11 parents were collected in 2015 from two regional varietal sites at Childers and Booyan. In the same year, seeds were germinated and grown in the nursery over two years. Seedling progeny were planted in four growers' trial sites at Childers, Alloway, Pine Creek and Emerald from 2017 to 2018. We will visually score yield on a 0 (none) to 10 (high) scale in January and February each year. Sample hand harvesting will be used to calibrate the rating scale. Kernel recovery (KR) will be estimated at age five. Advanced quantitative genetics methods will be used to predict breeding and clonal values as described in MC09021. Final evaluation will occur in 2024 and 2025 and will involve nut-in-shell (NIS) weights, full kernel and agronomic assessment of all elite selections. We will compile the early generation data on these elite selections and arrange for planting in regional variety trials via the rolling front strategy that we developed in MC17006.

1.2.3 B2.3 high density population (see Appendix 1.2.3)

For B2.3, we planted 1,617 open-pollinated progeny of 17 parents from a supplementary grower RVT trial at Decortes, QLD. Creation of this population will allow rapid phenotypic evaluation of the high heritability traits of precocity and KR followed by genomic selection for NIS yield. These seedling progeny were planted in 2018 at MRF at 5,000 trees ha^{-1} and may be the highest density fruiting orchard in the world. Nuts were harvested in April and planted in November 2018 representing a substantial reduction in seedling propagation time to only 6 months compared to 24 months in the B1.1 and B1.2 populations. In 2022, we expect trees to flower. The resulting crop will be evaluated in 2023 for KR and all non-fruiting trees will be culled. Progeny with KR over 40% will be evaluated using genomic selection for NIS yield (and other traits). These elite trees will be propagated in 2023 for secondary testing on grower properties. The original seedling trees will be consolidated by transplanting and will form the basis of a future polycross population.

Figure 1.2.3.1 Progeny trees planted in a high density (4m x 0.5 m) trial at the Maroochy Research Facility, Nambour

1.3 RVT3 diallel pollination experiment (see Appendix 1.3)

To identify suitable cross-compatible combinations, we have conducted hand pollination experiments on 19 elite RVT3 selections. We evaluated cross compatibility in the 2016 and 2017 growing seasons in a supplementary grower RVT3 trial planted at Alloway, QLD. In each year, we randomly selected three trees from each trial. Each tree was cross-pollinated with two pollen sources in 2016 ('HAES741' and 'HAES344') and three pollen sources in 2016 ('HAES741', 'HAES344' and 'HAES246'). Due to the variability in synchronization in flowering, only 11 genotypes (out of 19) could be cross-pollinated in 2016 and eight in 2017. For each cross, 15 randomly selected racemes per tree were bagged at looping stage of flowering to stop potential outcrossing. At anthesis (3-7 days after bagging), the bags were removed carefully and pollen from each raceme were collected by sliding a clean pollen tube over the raceme. Soon after the self-pollen was collected, the raceme was crossed with the pollen of the male parent collected in a separate pollen tube and the raceme was re-bagged to avoid any chance of contamination. At 90 days post-pollination, the paper bags were replaced with netted bags to hold the nuts until final data recording. All the trees under evaluation for cross-compatibility were also evaluated for nut setting under open pollination.

1.4 DNA technology to reduce breeding cycle time (see Appendix 1.4)

Genetic gain for yield may be increased, compared to traditional breeding approaches, through the use of strategies including (i) indirect selection using yield component traits, (ii) marker-assisted selection and (iii) genomic selection. A PhD study employed an experimental population of 295 seedling progeny and their 29 parents, at four sites across

south-east Queensland, that were genotyped for 4,113 SNPs and 16,171 silicoDArT markers detected using Diversity Arrays Technology (DArT) methods. Population structure, genetic diversity and linkage disequilibrium (LD) between SNP markers was quantified to inform subsequent genomics analysis (see Appendix 1.4.1). Flowering and nut characteristics, tree growth and yield were measured on each tree. Estimations of trait heritability and genetic correlations between each component trait and yield were used to calculate selection efficiency of indirectly selecting for yield using component traits (see Appendix 1.4.2). A genome-wide association study (GWAS) was used to identify genetic markers associated with component traits that were genetically correlated with yield or are considered economically important (see Appendix 1.4.3). Genomic selection (GS) used markers across the whole genome to predict estimated breeding values for yield and yield stability of individuals. Genetic gain was compared among traditional breeding methods and genomic selection methods (see Appendix 1.4.4).

2. Rootstocks and reduced tree size (see Appendices 2.1 and 2.2)

This section was a part of "transforming subtropical tree crop productivity", AI13004 led by Dr John Willkie. Aiming to identify potentially useful rootstocks for scion yield and vigour control, we propagated 30 genotypes of seedlings and cuttings from diverse origin and sources (Table 1 in Appendix 2.1) in April-October 2014. In July 2016, a commonly grown scion 'HAES741' was grafted onto the rootstocks, and the trial was planted on 4th April 2017 following the procedure described in Appendix 2.1. We phenotyped for growth characteristics and flowering of both scion and rootstock in November 2017, May 2018 and May 2019. Phenotyping will be continued until final measurements will be taken at age 7 in 2024. Additionally, we conducted an investigation on vascular systems to identify vascular traits associated with reduced tree size, and published an article on "Anatomical structures associated with vegetative growth variation in macadamia" in Plants & Soil (Appendix 2.2).

3. Economic analysis and extension to growers (see Appendix 3)

Cost Benefit Analysis

The Financial planner for macadamia was used to compare the relative economic performance of recent releases from the macadamia breeding program and regional variety trials. These 20-year cash flow forecasts compare both annual net cash flow and cumulative cash flow for the four recent variety releases (G, J, P and R) and compares these with established industry varieties A16 and 741. Forecasts included performance in both the Bundaberg region and the Northern Rivers of NSW. All scenarios model establishment of a new 100-hectare farm. Irrigation establishment and operating costs are included in all Bundaberg scenarios and excluded from all Northern Rivers scenarios. The industry average planting density 312 trees per hectare (8m x 4m) is assumed for all varieties other than P. The higher density of 400 trees per hectare for variety P is appropriate only for the Bundaberg region (not for NSW) due to the relatively small size of these trees. This analysis was undertaken to evaluate elite selections from the B1.1 populations in regional variety trials.

Communication strategy

A communications strategy was developed at the beginning of the project to maximise the understanding of the breeding program and adoption of the improved cultivars. The strategy included a range of communication methods including presentations at industry meetings, field days and farm walks at breeding sites, targeted articles in industry media, evaluation surveys and direct communication with growers, consultants and members of MIVIC.

Survey of grower co-operators

A survey of previous grower co-operators involved in progeny field trials was carried out in 2016. Eight owners and/or managers were surveyed about their experiences with breeding progeny field trials under their management.

The owners and managers were also surveyed about their willingness to participate in future trials and if so, how these trials could be better organised to suit their needs.

4. Pathology and entomology breeding research (see Appendix 4)

Aiming to screen a large number of diverse genotypes, in 2017 we converted the precocity trial (BNAMB11) into a husk spot (*Pseudocercospora macadamiae*) disease-screening nursery. We conducted experiments in October 2017 and October 2018 using an established screening procedure. Husks were collected from diseased trees and hung over trees chosen to be evaluated. Due to the variability in precocity, we evaluated 105 accessions representing 17 cultivars and 28 progeny families in 2017-2018 fruiting season. In 2018-2019 season, we evaluated 385 plants representing 32 families and 25 parents. Using overhead sprinklers, a moist environment was created to ensure spreading and infection of the disease to developing husks. Nots in infected trees were assessed for chlorotic flecking and premature nut drop. For further investigation on husk spot genomics, a PhD research project commenced in January 2019. As a part of the PhD project, we additionally set up husk spot screening experiment at the Tiaro ex-situ germplasm block in spring 2019 to evaluate 212 wild accessions and 14 cultivars. This wild accession screening will be repeated in 2020 season. Entomology breeding research was removed from the project, in consultation with Hort Innovation and the Australian Macadamia Society, due to budget constraints.

5. Wild germplasm trials and development (see Appendix 5)

The materials for this study were collected from two *ex situ* germplasm field trials in Tiaro QLD and Alstonville, NSW. These collections were established in 2000 and 2001, respectively, as the National Macadamia Germplasm Collection (NMGC). A total of 304 wild accessions were genotyped for molecular markers by DArT, and 540 trees were phenotyped for various nut, flower and tree growth traits from 2017 to 2019. A genetic diversity and population structure was conducted on all the accessions genotyped. A genome-wide association study will be performed to identify molecular markers associated with key breeding traits.

Outputs

1. Second generation of progeny

1.1 Populations produced by hand pollination (see Appendix 1)

We generated and planted 2,711 progeny from 151 families using 34 parents (Appendix 1.1.1 and 1.1.2). We have selected and propagated 5 progeny from BQBRS12 and BQBRS13 trials (B2.1 population). The list of the selected progeny is presented in Appendix 1.1.4. Based on evaluations to 2019, these progeny showed potentiality to produce >30% higher yield at age 6/7 than that of existing precocious cultivar 'A4'.

Table 1.1.2 Progeny information of B 2.1 population

Location	Year planted	Year germinated	#progeny planted	#Clones	#Standards	#Family	#Parents	Total	Comments
BQBRS	2012	2010	472	59	23	34	20	531	2 Elites selected in 2019
BQBRS	2013	2011	873	66	21	47	33	939	3 Elites selected in 2019
BQBRS	2014	2012	563	101	29	41	31	664	
BQBRS	2015	2013	553	95	45	29	25	648	
BQBRS	2016	2014	250	14	14	16	18	264	
Total			2711	335	88	136	64	3046	

1.2 Large populations at reduced cost (see Appendix 1.2)

We generated and planted 6,117 open pollinated seedlings. Seedlings are being evaluating using three breeding strategies: i) selection index for highly heritable traits(B2.2 precocity), ii) conventional breeding as used in MC09021 with visual scoring (B2.2), and iii) combination of selection index and genomics selection (B2.3) (Table 1.2.1).

Table 1.2.1 Progeny information of B2.2 and 2.3 progeny generated at reduced costs

Generation code	Location	Year planted	# progeny planted	#Clones	#Standards	#Family	#Parents	Total
B 2.2_Precocity	BNAMB	2011	704	282	27	39	39	986
B 2.2	BALLO	2017	1325	148	39	11	11	1473
	BPINE	2017	620	70	36	9	9	690
	BCHIL	2017	572	65	14	6	6	637
	BEMER	2018	575	118	43	7	7	693
	Total		3796	683	56	11	11	4479
B2.3	BNAMB	2018	1617	82	11	17	17	1699
	Total		1617	82	11	17	17	1699
	Grand total		6117					7164

1.2.1 B2.2 Precocity (see Appendix 1.2.1)

We have selected and propagated 18 precocious progeny from BNAMB11 trial. The list of the selected progeny is presented in Appendix 1.2.1.

1.2.2 B2.2 Polycross (see Appendix 1.2.2)

We generated and planted 3,796 open pollinated breeding progeny from 11 parents. Populations were planted in four trial sites in QLD (Table 1.2.1).

1.2.3 B2.3 high density progeny (see Appendix 1.2.3)

We generated and planted 1,617 open pollinated progeny at very high density (Table 1.2.1). This trial also included 82 clones of parent trees and 11 standard cultivars. Additionally, we reduced the nursery management cost by planting the seedings within six months of germination rather than the conventional 24 months. We also reduced around 80% cost of evaluation through high density planting (5000 seedling /ha) and shortening the progeny evaluation cycle into half (5 years).

1.3 RVT3 diallel pollination experiment (see Appendix 1.3)

We prepared a report showing the chart of self- and cross-compatibility (Appendix 1.3.1). Nuts produced from the diallel pollination experiment are being used in the naturally nutritious project (HN15001). Additionally, we identified that 14 out of 19 RVT3 selections showed some degree of self-compatibility and four of them had a significant level of self-compatibility. The highly significant self-compatible elites were “K”, “Q”, “D” and “A”. In 2016, Howell et al. presented a poster at the International Horticultural Congress. One article on nut-setting has been published in Acta Horticulturae (Howell et al, 2018). A synopsis of the article is presented in appendix 1.3.2.

1.4 DNA technology to reduce breeding cycle time (see Appendix 1.4)

Dr Katie O’Connor’s research gives an indication of the population structure and genetic diversity of the breeding program (see Appendix 1.4.1), and advances our understanding of the genetic control of yield component traits(see Appendix 1.4.2). The identification of QTLs for key traits is a useful resource and will provide a stepping-stone towards marker-assisted selection (MAS) in the future (see Appendix 1.4.3). This study was the first to assess the prospect of employing genomic selection (GS) in macadamia. To our knowledge, it is also the first to use GS to predict yield stability for a nut tree crop. This study developed simple GS models that achieved moderate yield prediction accuracies, as well as some strategies to incorporate GS into the Australian macadamia breeding program in multiple ways (see Appendix 1.4.4).

As well as four published peer-reviewed journal articles, two articles were published outlining the PhD research and key findings in the Australian Macadamia Society News Bulletin Magazine. Dr O’Connor presented her work at the International Macadamia Research Symposium in September 2017 in Hawaii, the National Macadamia Industry Conference in 2018, and the TropAg conference in November 2019 in Brisbane, as well as various poster presentations, industry meetings and other events.

2. Rootstocks and reduced tree size (see Appendices 2.1 and 2.2)

Dr Ben Toft generated data on the effect of rootstocks on scion productivity, precocity and vigour. As a part of AI13004 project, Twenty-seven rootstocks were genotyped using DArT markers, and the genetic diversity is presented in Appendix 2.1. Rootstocks will be propagated after final selection in 2024. Based on a study of vascular systems, we suggested that the size of xylem vessel can explain the variability in plant size. Further investigation on large number of genotypes and the mechanism of xylem vessel mediated vigour control is required. Five journal articles and an AMS news bulletin were published. One oral presentation and three poster presentation were made

throughout the project. We also presented the updates of this section in AMS consultants annual meetings, STPHI webinar series, QAAFI staff and student meetings, MIVIC meetings, and Mid-term evaluation of MC14000.

3. Economic analysis and extension to growers (see Appendix 3)

Cost Benefit Analysis

An economic evaluation of the breeding program compared the net present value (NPV), internal rate of return (IRR), annual net cash flow (NCF) and yearly cash balance (YCB) of recently release varieties G, J, P and R with industry standard varieties A16 and 741 in both Bundaberg and Northern Rivers NSW. Based on available yield and kernel recovery data to date, analysis of the four newly released varieties has identified their long-term economic potential compared with industry standard varieties.

Communication strategy

Delivery of a communications strategy has resulted in extensive industry consultation and increased the understanding of the breeding project among growers and key industry stakeholders. Key outputs from the communications strategy are outlined below.

Field days and farm walks

Regional variety trial field walks Bundaberg (February 2016) and Alstonville (February 2016)

Regional variety trial field walks Bundaberg (2nd March 2017) Alstonville (23rd March 2017)

Growers evaluated the recently released, elite macadamia selections from the industry breeding program at Regional Variety Trial (RVT) field walks in March in Bundaberg and Alstonville.

Growers attending the Alstonville Field Walk in 2017.

Survey of grower co-operators

The survey of grower co-operators carried out in 2016 provided feedback on limitations and differences of grower progeny trials including trial establishment and maintenance and legal contracts. Feedback from grower co-operators included;

"The better performing trees showed how much room there was for improvement compared to the usual varieties."

"We are extremely interested in new varieties and see this as a part of industry evolution and are happy to be a part of this."

"The trial did not interfere with any other farm management. It generally integrated well with the rest of the farm."

The survey identified potential trial sites for future progeny trials. Co-operators also provided suggestions on ways to improve communication and management protocols for future trials.

"We would like to meet with the researchers prior to planting to develop protocols for planting, pruning and other management."

4. Pathology and entomology breeding research (see Appendix 4)

We generated data on the variability in husk spot susceptibility in a large number of genotypes (Appendix 4). We are continuing the screening for resistance on 212 wild accessions. Screening will be completed in 2021. Ms Jasmine Nunn commenced PhD research into the genetics of resistance to macadamia husk spot in January 2019, supported by a Commonwealth scholarship. Two posters were presented by Jasmine Nunn in Tropag19 and Australasian Plant Pathology Society conferences.

5. Germplasm trials and development (see Appendix 5)

Ms Thuy Mai was awarded a travel grant for her PhD research and presented a poster titled "Genetic characterisation of wild macadamia germplasm" at the Plant and Animal Genome Conference in January 2019 in San Diego, California. A poster was also presented on Ms Mai's research by Dr Topp at the International Horticultural Congress in August, 2018 in Istanbul, Turkey, titled "Towards re-sequencing the whole genome of wild macadamia species: Selection of reference genome accessions based on molecular and phenotypic characterization**".**

Outcomes

1. Second generation of progeny

1.1 Populations produced by hand pollination

The five selected progeny are selected for precocity, kernel recovery, reduced growth and yield performance. The selected progeny showed potentiality of producing 35-200% higher yield at age 6-7 than that of existing precocious variety 'A4'. Therefore, these selections are expected to contribute to early farm profitability at >35% rate than the existing commercial varieties.

1.2 Large populations at reduced cost

The 18 selected progeny are selected for precocity, kernel recovery, reduced height. We expect, the selected progeny will increase the farm profitability at >30% through early production, high kernel recovery and reduced management cost.

1.3 RVT3 diallel pollination experiment

Planting the newly released varieties along with the suggested cross compatible cultivars can be useful to increase farm productivity. We also identified four self-compatible elites, which will contribute to the profitability of future macadamia industry, particularly if cross-pollination success is hampered due to climate change. An unexpected result from this research was the very high number (14 of 19) of elite RVT3 selections that had some degree of self-fertility. It indicates that there may be indirect selection for self-fertility when selecting directly for high yield. Further research is planned in MC19000 to examine this hypothesis. If true, then self-fertility may be an important trait in our future breeding and selection.

1.4 DNA technology to reduce breeding cycle time

Outcomes of Katie's PhD project include an understanding of the genetic diversity and population structure of the breeding population to inform future cross-pollinations. The genomics tools will lead to more rapid release of cultivars to industry due to the selection of elite candidates several years earlier than previously possible using traditional phenotyping techniques. This will lead to increased efficiency of breeding due to the reduction in time and hence cost of field maintenance and labour. Finally, quicker release of better varieties to industry, with the added goal of breeding precocious trees will lead to earlier returns on capital investments for growers and higher yields and associated income.

2. Rootstocks and reduced tree size

Xylem vessel size can be used as tool of early selection of breeding progeny, which will reduce cost of evaluation of large number of progeny in the field.

3. Economic analysis and extension to growers (see Appendix 3)

Evaluation of the economic performance of new varieties has generated information that will aid the adoption of the recently released varieties from the breeding program. Twenty-year cash flow forecast identified the performance of varieties G, J, P and R compared with industry standards. This analysis has provided growers and stakeholders with information that will assist selection of varieties for new plantings and tree/orchard replacement.

The communication strategy linked extension activities of the breeding program with growers, stakeholders and other R&D projects. Farm walks and news bulletin articles provided growers with information on the progress of new varietal development. The involvement of growers in progeny trials and evaluation of elite selections at field

walks has resulted in a high level of industry engagement throughout the project.

The survey of grower co-operators has resulted in improvements to grower progeny trials through improved communication and management strategies. Evaluation and feedback on a range of management practices associated with progeny trials has resulted in a refined protocol for grower co-operator sites and has identified additional collaborators for new progeny plantings.

4. Pathology and entomology breeding research (see Appendix 4)

No outcomes at this early stage of the research.

5. Germplasm trials and development (see Appendix 5)

Characterisation of the germplasm trials, encompassing accessions from all four species and admixture populations, will inform the breeding program. Genetic analysis will determine how related the wild germplasm is to the current breeding population, and will remain a vital source of genetic diversity for the future of the breeding program. Phenotypic analysis will inform the potential to introduce desired characteristics from wild accessions into the breeding program. For example, trees of *M. ternifolia* and *M. jansenii* are much smaller in structure than *M. tetraphylla* and *M. integrifolia*, and therefore could be incorporated into cross-pollinations to combine desired phenotypes for the development of small trees with high yield efficiency. Wild germplasm may also have resistance to pathogens, such as stick-tights, or compact nut drop pattern, both of which are desired traits for the breeding program.

Monitoring and evaluation

Our monitoring and evaluation was obtained by direction of a project steering committee (MIVIC) and by a mid-project review. Details of both are provided in the following section.

Meetings and workshops

MIVIC Meetings (Macadamia Industry Varietal Improvement Committee)

A commercialisation meeting was held in May 2015 to discuss the project plan for grower co-operator trials with industry.

A MIVIC meeting was conducted on 20 May 2015 as an online webinar. Reports were presented to the committee on the completion of breeding project MC09021 and the commencement of second generation breeding project MC14000.

A MIVIC meeting was held at Bundaberg on 23-24 September 2015, and results from breeding project were presented.

A MIVIC meeting was held on 19 February 2016 at Wollongbar. The breeding program was discussed and reported on by the project team but the majority of the meeting was concerned with the final management plans required for the RVT project.

A MIVIC meeting was held at Maroochy Research Station on 1 December 2016 with representatives from industry (6), HIA (2), QAAFI (2) DPI (1) and DAF (6). An update on the breeding project was presented but the main agenda item was the selection of four superior selections for release to industry in 2017. A report on advancing the industry breeding program by use of genomics was also presented.

A MIVIC meeting was held as a phone meeting on 17 October 2017, and a progress update of the breeding program was presented.

Results of the husk spot disease nursery experiment were reported at the MIVIC meeting in February 2018. An update on the breeding program was provided by Dr Bruce Topp, and the results of the mid-term review were presented at the meeting by Dr Vino Rajandran.

A MIVIC meeting was held as a phone meeting on 12 September 2018, and a progress update of the breeding program was presented

A MIVIC meeting was held at Nambour on 26 February 2019 and a report on the breeding project was presented. We took the opportunity to show MIVIC members two of our trials planted at Maroochy Research Facility.

Presented an outline of the newly funded MC19000 breeding project at the phone meeting with the MIVIC on 3rd September 2019.

Mid-project Review

A mid-project review (see appendices 6.1 and 6.2) was conducted from 27 November to 1 December 2017. On 27 November nine breeding team members presented seminars to Dr Jose Chaparro (reviewer), Dr Vino Rajandran (Hort Innovation R&D Manager), Mr David Bell (MIVIC Chair) and Mr Lindsay Bryen (SIAP member). The reviewer travelled to Bundaberg, Gympie, Nambour and Lismore to interview eight industry representatives who provided background information and context of the breeding program.

Other meetings/presentations/seminars

Topp, B. Presentation on breeding projects at HIA commercialisation meeting in May 2015.

Topp, B. Presentation on breeding projects at AMS Consultants Workshop, Brisbane, 10 June 2015.

Topp, B. Seminar on Plant Breeding. University of Queensland, June 2015

Two presentations at DAF Genetic Improvement Workshop, Nambour, 16 June 2015 on rootstock evaluation and scion breeding.

Topp, B. Invited presentation on the breeding project to the AMS Board, Bundaberg, 13 July 2015.

Alam, M. Rootstock screening. Small Tree High Productivity Initiative Review Meeting, November 2015

Breeding team. Macadamia breeding presentation to University of Sunshine Coast and University of Queensland students, yearly from 2015 to 2019.

Participated in Macadamia Conservation Council meeting in Brisbane on 1 March 2016.

Organised and participated in a workshop to determine an efficient method of selecting the best performing varieties in the RVTs. "Economic Weights workshop" held at Nambour on 4 March 2016.

Topp, B and Alam, M. Participated in meeting at Southern Cross University on genomic collaboration with Dr Kilian and Dr Nock on 30 March 2016.

Breeding team. Presentations on the macadamia breeding project were delivered at the Australian Macadamia Society's conference in the "speed dating the researcher" session at Caloundra on 19 October 2016, the small tree initiative annual meeting at Mareeba on the 26 October 2016 and the QAAFI annual meeting at Brisbane on 15 November 2016.

O'Connor, K. Cracking the code: Using DNA to find the top crop of macadamias. Three minute thesis competition, QAAFI and UQ, 2017

Topp, B. Presentation on breeding program at AMS consultant's forum, Brisbane, 7 June 2017

Toft, B. Macadamia architecture. Australian Macadamia Society Consultants Forum, Brisbane, June 2017

Propagation workshop for native plant nurseries was organised and conducted at Maroochy Research Facility on 8 August 2017 in conjunction with the Macadamia Conservation Trust, DAF and Healthy Land and Water.

O'Connor, K. Using DNA to find the top crop of macadamias. Future of Horticulture: Hort Connections, Brisbane, June 2018

Mai, T., Searching for better macadamias. Three minute thesis competition, QAAFI and UQ, 2018

Topp, B. Participation at two day workshop for the National Tree Genomics project, Brisbane, November 2018

Toft, B. Limb bending and canopy structure. Australian Macadamia Society Mac Group meetings (x6), Queensland and New South Wales, February – March 2019

Topp, B. and Mai, T. Field walk presentation through ex-situ macadamia wild germplasm trial at Tiaro, Queensland. July 2019

Invited presenter at the Macadamia Conservation Trust meeting at Nambour on 23 August 2019 and advised on propagation and diversity study.

Breeding team. Meeting, presentations and field walk with UQ/QAAFI colleagues. September 2019.

Topp, B. and O'Connor, K. Genomics in macadamia breeding. National Tree Genomics Project Annual Meeting, Brisbane, November 2019

Recommendations

Increase progeny field trial population sizes

The polycross method of producing large numbers of progeny trees should be employed to produce 5-10,000 seedling trees in the next 5-year project. Elite first and second generation selections that are planted for regional evaluation can be used as seed orchards for future polycross progeny production. While the polycross method should be used to produce the bulk of the seedling population, it will be important to conduct controlled hand-pollination to combine specific parents in complementary mating designs as required.

Breed for traits that will be "game-changers"

Emphasis on the grower-defined priority traits of NIS-yield, kernel recovery, tree size, disease resistance and quality will continue to be the main selection traits in the next project. However, other traits that may dramatically improve orchard profitability should also be examined. These include self-fertility as described in this report being linked to increased yield; compact nut-drop to allow for improved harvesting efficiency, and smaller trees with high yield efficiency to allow for improved spray penetration and reduced tree pruning management.

Examine methods to efficiently select for multiple traits

The bio-economic model and selection index used in MC09021 and MC14000 should be re-examined as improved industry confidence in the use of this model is required. A gap analysis to define trait priorities and models of trait expression should accompany developing quantitative biological models for traits such as precocity, tree vigour, nut-drop pattern, and husk-spot resistance. Other evaluation methods such as preference choice mapping should be examined as approaches to determine weightings of traits.

Examine the prospect of incorporating genomic selection tools to increase breeding efficiency

The marker-trait associations detected in Dr O'Connor's study should be further explored and validated in a separate study. GWAS can also be used to identify marker-trait associations for other important traits, such as self-fertility and resistance to pests and diseases. Combined with the completion of a macadamia reference genome, the locations of these markers on the genome can be confirmed and used to identify causal genes. The genomic selection models for yield need to be validated in a separate population, preferably with more individuals to represent wider phenotypic variance, and with more genetic markers to ensure that the small-effect genes controlling yield are captured by LD. Genomic selection models could also be constructed for other economically important traits that may be controlled by many genes, such as trunk circumference or tree size. Multi-trait genomic prediction models may improve the accuracy of prediction by incorporating more data, including that for yield component traits. Furthermore, the results of GWAS could be accommodated in genomic predictions. An economic analysis could compare the costs involved in genotyping a seedling progeny population with traditional breeding methods, and possibly alternative genotyping methods.

Rootstock and reduced tree size

We recommend to continue the existing rootstock screening trial. A molecular investigation can be useful to explore the mechanism of rootstock mediated regulation of scion growth and productivity. Investigating the vascular system over a large number of accessions can be helpful for rapid screening of reduced tree size. Genome wide association study can assist in identifying markers associated with tree vigour.

Pathology and entomology breeding research

We suggest to continue the screening of diverse germplasm including wild accessions and cultivars to identify potential sources of husk spot resistance in Macadamia. Genomic based investigation can be useful to identify marker associated with husk spot resistance, which can be utilized for rapid screening of seedling progeny in the nursery.

Refereed scientific publications

Scientific journal articles

Published

Akinsanmi O.A., Neal J., Drenth A., Topp B. (2016) Characterization of accessions and species of Macadamia to stem infection by *Phytophthora cinnamomi*. Plant Pathology 66:186-193

Akinsanmi O.A., Wang G., Neal J., Russell D., Drenth A., Topp B. (2016) Variation in susceptibility among macadamia genotypes and species to Phytophthora root decay caused by *Phytophthora cinnamomi*. Crop Protection 87:37-43

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Topp B., Hardner C., Neal J., Kelly A., Russell D., McConchie C., O'Hare P. (2016) Overview of the Australian macadamia industry breeding program. Acta Horticulturae 1127:45-50

Alam, M., Howell, E. and Topp, B. (2018) Variation in precocity in a macadamia breeding population. Acta Horticulturae. 1205:645-652

Alam, M., Neal, J., Howell, E., Russell, D. and Topp, B. (2018) 'MPM1': A macadamia hybrid showing breakdown of biological rhythm in morphogenesis. Acta Horticulturae 1205:631-636

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Alam, M., Wilkie, J. and Topp, B. (2018) Early growth and graft success in macadamia seedling and cutting rootstocks. Acta Horticulturae. 1205:637-644

Howell, E., Russell, D., Alam, M. and Topp, B. (2018) Variability of initial and final nut setting in macadamia superior selections through different pollination methods. Acta Horticulturae. 1205:617-622

O'Connor K.M, Hardner C.M., Alam M.M., Hayes B.J., and Topp B.L. (2018) Variation in floral and growth traits in a macadamia breeding population. Acta Horticulturae 1205(77):623-630

O'Connor K.M, Hayes B.J., and Topp B.L. (2018) Prospects for increasing yield in macadamia using component traits and genomics. Tree Genetics & Genomes 14:7

Toft, B., Alam, M. and Topp, B. (2018) Broad-sense heritability and inter-trait relationships in macadamia architecture, flowering and yield. Acta Horticulturae. 1205:609-616

Toft, B., Alam, M. and Topp, B. (2018) Estimating genetic parameters of architectural and reproductive traits in young macadamia cultivars. Tree Genetics & Genomes 14:50-59

Toft, B.D., Hanan, J.S., Topp, B.L., Auzmendi, I., Wilkie, J.D. (2018). Can greater understanding of macadamia canopy architecture lay the foundation for orchard productivity improvements? Acta Horticulturae 1228, 51-58

Alam, M.M., Hardner, C., Nock, C., O'Connor, K., Topp, B. (2019) Historical and molecular evidence of genetic identity of HAES 741 and HAES 600 macadamia cultivars. HortScience 54(4): 616-620

Hu, W., Fitzgerald, M., Topp, B., Alam, M. and O'Hare, T. (2019) A review of biological functions, health benefits, and possible de novo biosynthetic pathway of palmitoleic acid in macadamia nuts. Journal of Functional Foods 62:103520

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O'Connor, K., Kilian, A., Hayes, B., Hardner, C., Nock, C., Baten, A., Alam, M., and Topp, B. (2019) Population structure, genetic diversity and linkage disequilibrium in a macadamia breeding population using SNP and silicoDArT markers. Tree Genetics & Genomes 15(2): Article 24

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Toft, B.D., Alam, M.M. and Topp, B.L. (2019). Anatomical structure associated with vegetative growth variation in macadamia. Plant and Soil. 442

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Toft, B.D., Alam, M.M., Wilkie, J.D., Topp, B.L. (2019). Phenotypic association of multi-scale architectural traits with canopy volume and yield: moving towards high-density systems for macadamia. HortScience. 54(4), 596–602

In Press

Topp, B., Nock, C., Hardner, C., Alam, M. and O'Connor, K. Macadamia (*Macadamia* spp.) Breeding. In: J.M. Al-Khayri, S.M. Jain and D.V. Johnson (eds.), *Advances in Plant Breeding Strategies: Nut and Beverage Crops*, Vol. 4. Springer International Publishing, Switzerland. (Accepted, August 2018)

Submitted

O'Connor, K., Hayes, B., Hardner, C., Alam, M., Henry, R.J. and Topp, B. GWAS to identify genes associated with yield component traits in macadamia. BMC Genomics

In Preparation

O'Connor, K., Hayes, B., Hardner, C., Alam, M., and Topp, B. Variation, heritability and correlations of nut yield and component traits in Macadamia breeding progeny

O'Connor, K., Hayes, B., Hardner, C., Alam, M., Henry, R.J. and Topp, B. Genomic prediction accuracy for nut yield in the Australian macadamia breeding program

Mai, T., Alam, M., Hardner, R., Henry, R., and Topp, B. Population structure and genetic diversity in an ex-situ collection of wild macadamia germplasm. Proposed journal: Tree Genetics & Genomes.

Industry publications

Bignell, G., O'Hare, P. and Topp, B. (2016) Macadamia breeding progeny trials: What did we learn? Australian

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O'Connor, K. (2017) Improving macadamia yield using component traits and genomics. Australian Macadamia Society News Bulletin, Spring 2017, Volume 45, Number 3

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Toft, B., Alam, M., Topp, B. (2019). Limb bending and limb angle. Australian Macadamia Society News Bulletin, Summer 2019, Volume 47, Number 4

Conferences

Conference oral presentations

Toft, B. Can greater understanding of macadamia canopy architecture lay the foundation for orchard productivity improvements? XI International Symposium on Integrating Canopy, Rootstock and Environmental Physiology in Orchard Systems, Bologna, Italy, August-September, 2016

Alam, M., Howell, E., Hardner, C. and Topp, B. Precocity in Australian macadamia breeding progeny. International Symposia on Tropical and Temperate Horticulture, Cairns, Queensland, November 2016

Alam, M., Neal, J., Howell, E., Russell, D. and Topp, B. MPM1: A macadamia hybrid showing breakdown of biological rhythm in the morphogenesis. International Symposia on Tropical and Temperate Horticulture, Cairns, Queensland, November 2016

Toft, B. Broad-sense heritability and inter-trait relationships in young macadamia architecture, flowering and yield. I International Symposium on Tropical Plant Breeding, Cairn, Queensland, November 2016

O'Connor, K. Selecting for nut characteristics in macadamia using a genome wide association study. International Macadamia Research Symposium, Hawaii, September 2017

Toft, B. Making small trees: how important are architectural characteristics in macadamia? International Macadamia Research Symposium, Hawaii, September 2017

Topp, B. Opportunities and obstacles in macadamia breeding. International Macadamia Research Symposium, Hawaii, September 2017

Topp, B. Prospects for genetic improvement of macadamia. TropAg, Brisbane, November 2017

Alam, M. Australian macadamia breeding and future directions. 8th International macadamia symposium, Lincang, China, 2018

Hardner, C. History of genetic improvement on macadamia and the origin of commercial cultivars. 8th International macadamia symposium, Lincang, China, 2018

Akinsanmi, Femi.Fast track orchard productivity gain with attention to insidious Phytophthora in macadamia. 8th International macadamia symposium, Lincang, China, 2018

O'Connor, K. Using DNA markers to predict yield and nut characteristics in macadamia. Australian Macadamia Industry Conference, Gold Coast, November 2018

Toft, B. Exploring canopy architecture for improved yield efficiency. Australian Macadamia Society Industry Conference, Gold Coast, November 2018.

Alam, M and O'Connor, K. Breeding Macadamia cultivars for orchards of the future. TropAg, Brisbane, Australia, November 2019

Alam, M and Shiddiky, M. State of art technologies for the improvement of macadamia: prospects and challenges. International Macadamia Annual R&D Conference 2019, Lincang, China, November 2019

Hardner, C. Wild origins of domesticated macadamia germplasm identified through chloroplast sequencing. International Macadamia Annual R&D Conference 2019, Lincang, China, November 2019

Topp, B. **Session chair.** Future Orchards: Advances in horticultural tree research. TropAg, Brisbane, Australia, November 2019

Topp, B. **Session chair.** Macadamia breeding. International macadamia annual R&D conference, Lincang, China, November 2019

Topp, B. Updates on Australian national macadamia breeding program. International macadamia annual R&D conference, Lincang, China, November 2019

Wilkie, J. Understanding early orchard productivity in macadamia. TropAg, Brisbane, Australia, November 2019

Conference poster presentations

Neal, J, Kelly, A., Hardner, C. McConchie, C. and Topp, B. Evaluating macadamia rootstocks. Tropag15, Brisbane. November 2015

O'Connor, K., Hayes, B., Hardner, C., Alam, M., Kilian, A., Topp, B. Prospects for genomic selection in macadamia, an outcrossing perennial rainforest tree. V International Conference on Quantitative Genetics ICQG5, Wisconsin, USA, June 2016

Alam, M., Wilkie, J. and Topp, B. Early growth and graft success in macadamia seedling and cutting rootstocks. International Symposia on Tropical and Temperate Horticulture, Cairns, Queensland, November 2016

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Alam, M., Hardner, C., Nock, C., O'Connor, K. and Topp, B. Historical and molecular evidence of genetic identity of 'HAES 741' and 'HAES 660' macadamia cultivars. International Macadamia Research Symposium, Hawaii, September 2017

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Alam, M., Mai, T., and Topp, B. Flowering time in a diverse range of macadamia cultivars grown in Australian climatic condition**.** International Horticultural Congress, Istanbul, Turkey, August 2018

Mai, T., Alam, M., Hardner, C., Henry, R., and Topp, B. Towards re-sequencing the whole genome of wild macadamia species: Selection of reference genome accessions based on molecular and phenotypic characterization**.** International Horticultural Congress, Istanbul, Turkey, August 2018

O'Connor, K., Hayes, B., Hardner, C., Henry, R., Alam, M., Topp, B. Genomic prediction for nut yield in a macadamia breeding population using GBLUP. Queensland Alliance for Agriculture and Food Innovation Research Meeting, Brisbane, November 2018

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Russell, D., DeFaveri, J., Hardner, C., Topp, B., Bell, D., Mulo, S. and Bignell, G. Four new macadamia varieties for the Australian industry. TropAg, Brisbane, November 2019

Nunn, J., Alam, M., Akinsanmi, O., Hardner, C., and Topp, B. Breeding for resistance to husk spot disease in macadamia. TropAg, Brisbane, November 2019

Nunn, J., Alam, M., Akinsanmi, O., Hardner, C., and Topp, B. Screening of a macadamia breeding population for resistance to husk spot. Australasian Plant Pathology Society Conference, Melbourne, November 2019

Intellectual property, commercialisation and confidentiality

Elite selections presented in appendices

- 1.1.3,
- 1.1.3.1,
- 1.2.1, and
- 1.2.1.1

are intellectual property produced in this project. Material Transfer Agreements have been used when these trees are planted on non-DAF sites.

Three of the appendices (1.3.1, 2.1 and 5.1) are confidential because they have not been submitted to journals for publication. They will be submitted in 2020 after which they will no longer be confidential. A third appendix (6.1 and 6.2), the mid-project review, has been marked as confidential by Hort Innovation.

No commercialization issues to report.

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Appendices

Appendix 1.1.1 List of parents for B2.1 progeny population

Parents	Number of progeny					
	2012	2013	2014	2015	2016	Total
816					13	13
849	48	184	2			234
A268		95	155	74		324
A376		59				59
A4	44	34	9			87
BALLO02-6-17				119	6	125
BALLO02-6-60				47	20	67
BALLO02-6-76				44	15	59
BAMAM02-2-3				37		37
BAMAM02-6-21				37		37
BAMAM02-6-3	17	2	4	4		27
BBAFF02-4-32			1			1
BBAFF03-15-24			1	12		13
BBAFF03-15-8		2	82			84
BBAFF03-21-29			107			107
BBAFF03-21-5				47		47
Beaumont	67	98	1	26		192
BEGYM01-12-3			47			47
BEGYM01-19-4			8			8
BHINK00-1-208				111	11	122
BHINK00-1-54		1	92		16	109
BHINK00-1-55			76		20	96
BQBRS01-12-12			1	169	15	185
BQBRS01-12-4					4	4
BQBRS01-13-4			1			1
BQBRS01-20-4		1	157		36	194
BQBRS03-11-6		93				93
BQBRS03-12-12				33	7	40
BQBRS03-12-4		65				65
BQBRS03-12-6			1			1
BQBRS03-13-19					4	4
BQBRS03-34-10				103	6	109

Parents	Number of progeny					
	2012	2013	2014	2015	2016	Total
BQBRS03-37-8				16	13	29
BQBRS97-2-46	94	60				154
BQBRS98-10-101	22	34	4			60
BQBRS98-10-111		9	13			22
BQBRS98-10-93		54				54
BQBRS98-11-80	11	2				13
BQBRS98-13-115	3	20	29			52
BQBRS98-14-25	53	110	33			196
BQBRS98-14-93	63	12				75
BQBRS98-16-37		39	3	4		46
BQBRS98-16-41	46	32				78
BQBRS98-4-73		16				16
BQBRS98-5-74		5				5
BQBRS98-6-79	17	6	1		13	37
BQBRS98-7-74	21					21
BQBRS98-8-87	29	13				42
BTFRS98-11-1			2			2
BTFRS98-37-1		3	21	4		28
BTFRS98-43-23			27			27
Daddow	100	121				221
Fuji; HAES 791	144	273				417
GTIAR01-14-16		7	24	22		53
GTIAR01-7-11		9	41	26		76
Ian McConachie dwarf	36					36
Jansenii_MRF arboretum	10	1	1	1		13
Kau; HAES 344	9	32				41
Mauka; HAES 741		66	78	118	118	380
NG18			4	16	118	138
NG7					50	50
Release				16		16
Ternifolia_MRF arboretum	11			5		16
Wild M. tetraphylla_9002				2		2
Unknown tree			100	13	15	128

Appendix 1.1.2 List of families for B2.1 progeny population

Family (Female x Male)	Number of progeny					
	2012	2013	2014	2015	2016	Total
849 X A4	3					3
849 X Daddow	10	12				22
849 X Fuji; HAES 791	7	49				56
849 X Ian McConachie dwarf	5					5
849 X Jansenii_MRF arboretum			1			1
A268 X A268			1			1
A268 X BQBRS98-14-93		12				12
A268 X BQBRS98-16-37		11				11
A268 X BTFRS98-11-1			2			2
A268 X BTFRS98-37-1		1	1			2
A268 X GTIAR01-14-16		7	24	22		53
A268 X GTIAR01-7-11		9	41	26		76
A268 X Jansenii_MRF arboretum		1		1		2
A268 X Mauka; HAES 741			78	20		98
A268 X Ternifolia_MRF arboretum				5		5
A268 X Unknown tree		2				2
A376 X Unknown tree		59				59
A4 X 849		3				3
A4 X Daddow	21	5				26
A4 X Fuji; HAES 791	12	25				37
BALLO02-6-17 X BALLO02-6-60					6	6
BALLO02-6-17 X BALLO02-6-76				30		30
BALLO02-6-17 X BQBRS01-12-12				89		89
BALLO02-6-60 X BQBRS01-12-12				24		24
BAMAM02-6-21 X BAMAM02-2-3				37		37
BAMAM02-6-3 X 849			1			1
BAMAM02-6-3 X A4	8					8
BAMAM02-6-3 X BQBRS98-16-37			3	4		7
BAMAM02-6-3 X Unknown tree	6					6
BBAFF02-4-32 X BEGYM01-19-4			1			1
BBAFF03-15-24 X BQBRS01-12-12			1			1
BBAFF03-15-8 X BBAFF03-21-29			3			3
BBAFF03-15-8 X BHINK00-1-55			9			9
BBAFF03-15-8 X BQBRS01-20-4		1	7			8
BBAFF03-21-29 X BBAFF03-15-8			18			18
BBAFF03-21-29 X BHINK00-1-55			11			11

Family (Female x Male)	Number of progeny					
	2012	2013	2014	2015	2016	Total
BBAFF03-21-29 X BQBRS01-20-4			4			4
Beaumont X Beaumont				5		5
Beaumont X Release				16		16
Beaumont X Unknown tree	67	98	1			166
BHINK00-1-208 X BALLO02-6-60				23		23
BHINK00-1-208 X BALLO02-6-76				14	11	25
BHINK00-1-208 X BBAFF03-15-24				12		12
BHINK00-1-208 X BHINK00-1-208				3		3
BHINK00-1-208 X BQBRS01-12-12				56		56
BHINK00-1-54 X BBAFF03-15-8		1	38			39
BHINK00-1-54 X BBAFF03-21-29			6			6
BHINK00-1-54 X BEGYM01-12-3			15			15
BHINK00-1-54 X BEGYM01-19-4			7			7
BHINK00-1-54 X BQBRS01-20-4			6		16	22
BHINK00-1-55 X BBAFF03-21-29			20			20
BHINK00-1-55 X BQBRS01-20-4			8		20	28
BQBRS01-12-4 X Unknown tree					4	4
BQBRS01-20-4 X BBAFF03-15-8			7			7
BQBRS01-20-4 X BBAFF03-21-29			45			45
BQBRS01-20-4 X BEGYM01-12-3			32			32
BQBRS01-20-4 X BHINK00-1-54			20			20
BQBRS01-20-4 X BHINK00-1-55			28			28
BQBRS03-11-6 X A268		40				40
BQBRS03-11-6 X BQBRS03-12-4		53				53
BQBRS03-12-12 X BQBRS03-12-12				2		2
BQBRS03-12-12 X Mauka; HAES 741				20		20
BQBRS03-12-12 X Unknown tree				9		9
BQBRS03-12-4 X A268		12				12
BQBRS03-12-6 X BQBRS01-13-4			1			1
BQBRS03-13-19 X NG7					4	4
BQBRS03-34-10 X BBAFF03-21-5				47		47
BQBRS03-34-10 X BQBRS03-34-10				1		1
BQBRS03-34-10 X Mauka; HAES 741				42		42
BQBRS03-37-8 X BQBRS03-12-12					7	7
BQBRS03-37-8 X BQBRS03-34-10				12	6	18
BQBRS03-37-8 X Mauka; HAES 741				4		4
BQBRS97-2-46 X BQBRS98-10-93		45				45
BQBRS97-2-46 X BQBRS98-11-80		2				2
BQBRS97-2-46 X BQBRS98-14-25	31					31

Family (Female x Male)	Number of progeny					
	2012	2013	2014	2015	2016	Total
BQBRS97-2-46 X BQBRS98-16-41	41					41
BQBRS97-2-46 X BQBRS98-7-74	14					14
BQBRS97-2-46 X BQBRS98-8-87		6				6
BQBRS97-2-46 X Unknown tree	8					8
BQBRS98-10-101 X BQBRS98-14-25	17	28	3			48
BQBRS98-10-101 X BQBRS98-16-41		6				6
BQBRS98-10-101 X BQBRS98-6-79	5		1			6
BQBRS98-10-111 X Unknown tree		9	13			22
BQBRS98-11-80 X BQBRS98-8-87	11					11
BQBRS98-13-115 X Unknown tree	3	20	29			52
BQBRS98-14-25 X Fuji; HAES 791		57				57
BQBRS98-14-25 X Unknown tree			30			30
BQBRS98-14-93 X Unknown tree	7					7
BQBRS98-16-37 X BQBRS98-14-25		6				6
BQBRS98-16-37 X BQBRS98-8-87		2				2
BQBRS98-16-41 X BQBRS97-2-46		7				7
BQBRS98-16-41 X BQBRS98-14-25		19				19
BQBRS98-4-73 X BQBRS98-10-93		4				4
BQBRS98-4-73 X BQBRS98-16-37		12				12
BQBRS98-5-74 X BQBRS98-8-87		5				5
BQBRS98-6-79 X BQBRS98-10-93		5				5
BQBRS98-6-79 X BQBRS98-16-37		1				1
BQBRS98-6-79 X BQBRS98-6-79					1	1
BQBRS98-6-79 X Unknown tree					11	11
BQBRS98-7-74 X BQBRS98-6-79	7					7
BQBRS98-8-87 X BQBRS98-14-25	5					5
BQBRS98-8-87 X BQBRS98-6-79	5					5
BQBRS98-8-87 X Unknown tree	8					8
BTFRS98-37-1 X A268			7			7
BTFRS98-37-1 X A4			9			9
BTFRS98-37-1 X BAMAM02-6-3		2				2
BTFRS98-37-1 X BTFRS98-37-1			2			2
BTFRS98-37-1 X Unknown tree				4		4
BTFRS98-43-23 X Unknown tree			27			27
Daddow X 849		29				29
Daddow X Fuji; HAES 791	15	32				47
Daddow X Kau; HAES 344		10				10
Fuji; HAES 791 X 849	14	87				101
Fuji; HAES 791 X BAMAM02-6-3	1					1

Family (Female x Male)	Number of progeny					
	2012	2013	2014	2015	2016	Total
Fuji; HAES 791 X BQBRS98-14-93	41					41
Fuji; HAES 791 X BQBRS98-16-37		7				7
Fuji; HAES 791 X Daddow	54	16				70
Ian McConachie dwarf X 849	9					9
Ian McConachie dwarf X BAMAM02-6-3	2					2
Ian McConachie dwarf X BQBRS98-14-93	15					15
Ian McConachie dwarf X BQBRS98-16-41	5					5
Jansenii_MRF arboretum X Ternifolia_MRF arboretum	6					6
Kau; HAES 344 X 849		4				4
Kau; HAES 344 X A4		1				1
Kau; HAES 344 X Daddow		17				17
Kau; HAES 344 X Jansenii_MRF arboretum	4					4
Mauka; HAES 741 X Mauka; HAES 741		33		16		49
Mauka; HAES 741 X NG18					113	113
NG18 X Mauka; HAES 741					5	5
NG18 X NG18			2	8		10
NG7 X 816					13	13
NG7 X BALLO02-6-60					14	14
NG7 X BALLO02-6-76					4	4
NG7 X BQBRS01-12-12					15	15
*M. ternifolia*_MRF arboretum X Kau; HAES 344	5					5
Wild *M. tetraphylla*_9002 X Wild *M. tetraphylla*_9002				1		1

Appendix 1.1.3: Selecting breeding progeny from BQBRS12 and BQBRS 13 trials based on a selection index

Mobashwer Alam, Craig Hardner and Bruce Topp

Introduction

Macadamia trees in commercial orchards grow to a height of 12-15 m and may take 15 years to attain peak production. The large size and slow maturity of the trees pose problems for breeding and selection. Topp et al. (2012) proposed a two-stage (tandem) breeding strategy that finishes two to three years earlier than the strategy currently employed by the Macadamia Breeding Program. Here, we considered precocity, tree size and kernel recovery as key traits of selection. Precocity indicates the early flowering and early fruiting/nut bearing in a tree, and assists in shortening breeding cycle through reducing juvenility, a major impediment of tree breeding (Mehlenbacher and Smith, 1992). In Eucalyptus, Chambers et al. (1997) described precocity as a highly heritable trait, while Hansche (1986) indicated precocity as a moderately heritable trait in peach. Development of a precocious macadamia variety having high kernel recovery can be useful for earlier economic returns to growers. Aiming this, we selected macadamia progeny from two breeding trials planted in 2012 (BQBRS12) and 2013 (BQBRS13).

Materials and methods

Planting and management of BQBRS12 and BQBRS13 trials

Two breeding trials were planted at Bundaberg research facility (BRF) in 2012 and 2013 growing seasons, named as BQBRS12 and BQBRS13. The trials consisted of 1345 (472 in BQBRS12 and 1345 in BQBRS13) progeny from 71 families (34 in BQBRS12 and 47 in BQBRS13) (Table 1.1.4.1). An incomplete block design was followed to accumulate this large number of single plant progeny, where 2-5 replications of standards were spatially distributed across the incomplete blocks. Standard macadamia management system was followed. Weeding and pesticides were applied whenever required.

Table 1.1.4.1. List of macadamia progeny families planted at Bundaberg research facility (BRF) in 2012 and 2013 growing seasons.

Sl no	Pedigree	2012	2013	Total
1	849 X A4	3		3
2	849 X Daddow	10	12	22
3	849 X Fuji; HAES 791	7	49	56
4	849 X Ian McConachie dwarf	5		5
5	A268 X BQBRS98-14-93		12	12
6	A268 X BQBRS98-16-37		11	11
7	A268 X BTFRS98-37-1		1	1
8	A268 X GTIAR01-14-16		7	7
9	A268 X GTIAR01-7-11		9	9
10	A268 X Jansenii_MRF arboretum		1	1

Sl no	Pedigree	2012	2013	Total
11	A268 X Unknown tree		2	2
12	A376 X Unknown tree		59	59
13	A4 X 849		3	3
14	A4 X Daddow	21	5	26
15	A4 X Fuji; HAES 791	12	25	37
16	BAMAM02-6-3 X A4	8		8
17	BAMAM02-6-3 X Unknown tree	6		6
18	BBAFF03-15-8 X BQBRS01-20-4		1	1
19	Beaumont X Unknown tree	67	98	165
20	BHINK00-1-54 X BBAFF03-15-8		1	1
21	BQBRS03-11-6 X A268		40	40
22	BQBRS03-11-6 X BQBRS03-12-4		53	53
23	BQBRS03-12-4 X A268		12	12
24	BQBRS97-2-46 X BQBRS98-10-93		45	45
25	BQBRS97-2-46 X BQBRS98-11-80		2	2
26	BQBRS97-2-46 X BQBRS98-14-25	31		31
27	BQBRS97-2-46 X BQBRS98-16-41	41		41
28	BQBRS97-2-46 X BQBRS98-7-74	14		14
29	BQBRS97-2-46 X BQBRS98-8-87		6	6
30	BQBRS97-2-46 X Unknown tree	8		8
31	BQBRS98-10-101 X BQBRS98-14-25	17	28	45
32	BQBRS98-10-101 X BQBRS98-16-41		6	6
33	BQBRS98-10-101 X BQBRS98-6-79	5		5
34	BQBRS98-10-111 X Unknown tree		9	9
35	BQBRS98-11-80 X BQBRS98-8-87	11		11
36	BQBRS98-13-115 X Unknown tree	3	20	23
37	BQBRS98-14-25 X Fuji; HAES 791		57	57
38	BQBRS98-14-93 X Unknown tree	7		7
39	BQBRS98-16-37 X BQBRS98-14-25		6	6
40	BQBRS98-16-37 X BQBRS98-8-87		2	2
41	BQBRS98-16-41 X BQBRS97-2-46		7	7
42	BQBRS98-16-41 X BQBRS98-14-25		19	19
43	BQBRS98-4-73 X BQBRS98-10-93		4	4
44	BQBRS98-4-73 X BQBRS98-16-37		12	12
45	BQBRS98-5-74 X BQBRS98-8-87		5	5
46	BQBRS98-6-79 X BQBRS98-10-93		5	5
47	BQBRS98-6-79 X BQBRS98-16-37		1	1
48	BQBRS98-7-74 X BQBRS98-6-79	7		7
49	BQBRS98-8-87 X BQBRS98-14-25	5		5
50	BQBRS98-8-87 X BQBRS98-6-79	5		5

Sl no	Pedigree	2012	2013	Total
51	BQBRS98-8-87 X Unknown tree	8		8
52	BTFRS98-37-1 X BAMAM02-6-3		2	2
53	Daddow X 849		29	29
54	Daddow X Fuji; HAES 791	15	32	47
55	Daddow X Kau; HAES 344		10	10
56	HAES 791 X 849	14	87	101
57	HAES 791 X BAMAM02-6-3	1		1
58	HAES 791 X BQBRS98-14-93	41		41
59	HAES 791 X BQBRS98-16-37		7	7
60	HAES 791 X Daddow	54	16	70
61	IMC dwarf X 849	9		9
62	IMC dwarf X BAMAM02-6-3	2		2
63	IMC dwarf X BQBRS98-14-93	15		15
64	IMC dwarf X BQBRS98-16-41	5		5
65	Jansenii_MRF arboretum X Ternifolia_MRF arboretum	6		6
66	HAES 344 X 849		4	4
67	HAES 344 X A4		1	1
68	HAES 344 X Daddow		17	17
69	HAES 344 X Jansenii_MRF arboretum	4		4
70	HAES741 X Mauka; HAES741		33	33
71	Ternifolia_MRF arboretum X HAES 344	5		5
	Total	472	873	1345

Data analysis

Following data checking for individual trait, multi-trial mixed model analyses were undertaken to estimate model parameters (i.e. genetic and residual variances) and predict genetic effects for each trait. Broad-sense heritability (i.e. index of the strength of genetic influence on a trait) was estimated as:

$$H = \frac{vA + vD}{vA + vD + vR}$$

where vA was the estimated additive genetic variance, vD was the estimated dominance genetic variance, and vR was the estimated non-genetic residual variance.

Model used in this analysis was-

$$y_{ij} = t_j + a_{ij} + d_{ij} + e_{ij}$$

where tj was the fixed effect of the j^{th} trial, a_{ij} was the random additive genetic effect of the i^{th} individual in the j^{th} trial, d_{ij} was the random dominance genetic effect of the i^{th} individual in the j^{th} trial, and e_{ij} was the random residual effect of the i^{th} individual in the j^{th} trial. The same model was used for cumulative nut mass, with observations square-

root transformed to achieve normality of residual distribution. The linear model for total kernel recovery was simplified by assuming genetic and residual effects were homogeneous across trials (i.e. genetic variance homogenous and genetic correlation equal to 1) and preliminary analyses suggested dominance was not significant for this trait, i.e.

$$y_{ij} = t_j + a_i + e_i .$$

Using the estimated model, additive and dominance values were predicted for each individual in each trial for the traits. These were summed to give clonal values (i.e. a+d), and the trial means from each trial were added. Predicted clonal values for cumulative nut mass on the square root scale were squared to express the clonal means on the scale of assessment.

Table 1.1.4.2 Summary of number and average of observations for canopy width (along and across planting row), total tree height, and skirt height, total nut mass per tree, and total kernel recovery in 531 planted trees in BQBRS12

Traits		**Assessment Year**				
		2015	**2016**	**2017**	**2018**	**2019**
HGT (m)	Mean	0.86	2.43	2.8		
	SD	0.33	0.64	0.61		
	Obs	472	529	462		
HGT_Sk (m)	Mean		0.63	0.62		
	SD		0.2	0.19		
	Obs		513	459		
CW (m)	Mean	0.86	1.79	2.46		
	SD	0.33	0.63	0.78		
	Obs	472	517	462		
CWa (m)	Mean		1.66	2.36		
	SD		0.54	0.7		
	Obs		517	462		
TNM (g)	Mean		160.8	551.63	1031.53	3035.55
	SD		130.64	656.97	1035.59	2216.55
	Obs		10	178	367	458
CNM19 (g)	Mean					3909.31
	SD					3009.97
	Obs					478
TKR18 (%)	Mean					36.76
	SD					5.08
	Obs					268

Results

Data summary of BQBRS12 and BQBRS13 trials

BQBRS12 and BQBRS13 trials were planted in a regular planting density (row X tree = 8 m X 4 m). For growth parameters, we considered all the growth parameters, including height (HGT), canopy width along the row (CW), canopy width across the row (CWa), and skirt height (HGT_Sk). Tables 1.1.5.2&3 summarise available data for analyses. Analyses was performed on canopy volume (estimated as π × cw × cwa × [hgt – SK_hgt]) at 2017 (VOL17), cumulative total nut mass to 2019 (CNM19), and total kernel recovery in 2018 (TKR18).

Table 1.1.4.3 Summary of number and average of observations for canopy width (along and across planting row), total tree height, and skirt height, total nut mass per tree, and total kernel recovery 939 planted trees in BQBRS13

Traits		**Assessment Year**				
		2015	**2016**	**2017**	**2018**	**2019**
HGT (m)	Mean	0.81	2.57	3.09		
	SD	0.38	0.66	0.64		
	#Obs	893	917	799		
HGT_Sk (m)	Mean		0.68	0.69		
	SD		0.22	0.22		
	#Obs		915	798		
CW (m)	Mean	0.81	1.76	2.55		
	SD	0.38	0.6	0.74		
	#Obs	893	908	799		
CWa (m)	Mean		1.82	2.67		
	SD		0.62	0.74		
	#Obs		908	799		
TNM (g)	Mean		420.08	687.18	1576.86	5083.42
	SD		334.31	847.65	1588.82	3211.25
	#Obs		19	331	709	774
CNM19 (g)	Mean					6235.85
	SD					4776.18
	#Obs					848
TKR18 (%)	Mean					38.21
	SD					5.38
	#Obs					566

Genetic parameters from BQBRS12 and BQBRS13 trials

Genetic parameters were estimated and presented in Table 1.1.5.4. Strong additive genetic correlations (rA=1) between sites was observed for TKR18, while the non-additive genetic correlation was null for the trait. Interestingly, the non-additive genetic correlation was negative for CNM19, and strongly positive for VOL17.

Table 1.1.4.4 Estimated genetic for three traits estimated by using linear models.

Genetic parameters	VOL17	CNM19	TKR18
vA@BQBRS12	0.127	275	13.4
vA@BQBRS13	0.174	542	13.4
rA@BQBRS12,BQBRS13	0.72	0.67	1.00
vD@BQBRS12	0.112	210	0
vD@BQBRS13	0.000	113	0
rD@BQBRS12,BQBRS13	0.99	-0.89	
vR@BQBRS12	0.531	327	17.9
vR@BQBRS13	0.780	553	17.9
h2@BQBRS12	0.16	0.34	0.43
h2@BQBRS13	0.18	0.45	0.43
H2@BQBRS12	0.31	0.60	0.43
H2@BQBRS13	0.18	0.54	0.43

Selection of elite progeny from BQBRS12 and BQBRS13 trial

To select precocious progeny with high kernel recovery and small plant size from BQBRS12 and BQBRS13 trials we developed selection index. The clonal values for total kernel recovery (gTKR, %) in 2018, cumulative nut-mass (gCNM, g) in 2019, and tree volume (gVOL) in 2017 were considered for the progeny selection process. To develop selection index, we considered trait heritability and economic importance of each trait, and the following equation was used to develop selection index for BQBRS12 and BQBRS13 (SI_{12_13}):

$$SI_{12\_13} = 0.43*gTKR\ (Standardized) + 0.55*gCNM - 0.25*gVOL\ (Standardized) \quad \text{Eq 1}$$

To get the selection indices for each progeny, we used the standardized clonal values of each trait. Based on the selection index values, we have selected 15 top ranked progeny from BQBRS12 and 25 from BQBRS13 trials. All these 40 (15+25) selections were evaluated for tree structure and health. For selecting the elite progeny, we considered the presence and level of disease infestation, bitterness, canopy structure, plant size and the level of stick tight. In addition, we also considered precocity of the progeny for selecting elites. Considering all the health and structure parameters, and precocity, we finally selected 2 progeny from BQBRS12 and 3 from BQBRS13 for next tier evaluation (Table 9). The selected seedling progeny showed a potentiality of providing 34-200 % higher yield at age 6-7 than that of existing precocious cultivar 'A4'. These elites were grafted onto H2 seedling rootstocks to include in the regional variety trial 4 (RVT4). The details of these elite selections are presented in Table S2.

1.1.4.5 Clonal values for Cumulative nut-in-shell yield, gCNM (g/tree); kernel recovery, gTKR (%); tree volume gVOL (cm), and selection index ranking, SI, of the selected progeny from BQBRS12 and BQBRS13 trials.

UQ code/ cultivar	Trials	gCNM (g)		gTKR (%)		gVOL (m3)		Selection index rank	
		BQBRS12	BQBRS13	BQBRS12	BQBRS13	BQBRS12	BQBRS13	BQBRS12	BQBRS13
UQM54	BQBRS12	11457		39		3.59		1	0
UQM55	BQBRS12	5008		43		3.26		2	0
UQM56	BQBRS13		10266		40		3.11	0	2
UQM57	BQBRS13		12154		39		3.22	0	1
UQM58	BQBRS13		10945		39		3.46	0	14
HAES344	BQBRS12	690		36		2.11			
A4	BQBRS12	3738		41		2.61			
HAES344	BQBRS13		5965		36		2.98		
A4	BQBRS13		6665		41		3.02		

Table 1.1.4.S1: Details of Elite selections from BQBRS12 and BQBRS13 trials

UQ code	Barcode	TreeID	Female	Male	Selection year	Comments
UQM54	BQBRS12-184-24	201014009	A4	791	2019	
UQM55	BQBRS12-187-22	201012003	849	A4	2019	
UQM56	BQBRS13-126-5	201117014	BQBRS03-11-6	A268	2019	One parent semi dwarf
UQM58	BQBRS13-132-61	201108045	849	791	2019	
UQM57	BQBRS13-133-25	201117035	BQBRS03-11-6	A268	2019	One parent semi dwarf

Table 1.1.4.S2: Summary of Elite selections from second generation breeding

Breeding code	Location	Year of planting	Year of selection	# Selection
B2.1	BQBRS	2012	2019	2
B2.1	BQBRS	2013	2019	3
B2.2	BNAMB	2011	2018	18

Appendix 1.1.3.1: Descriptions of elite selections from B2.1 progeny populations planted in BQBRS12 and BQBRS13 trials

A4

Standard

Female Parent	
Male Parent	

Sites	Raw Data		Genetic data	
	Trait	Data	Trait	Data
BQBRS12 Site	NIS 2017 (kg)	0.03	gCNM (kg)	3.74
BQBRS13 Site	NIS 2017 (kg)	0.35		6.67
BQBRS12 Site	NIS 2018 (kg)	0.67	gTKR (%)	41
BQBRS13 Site	NIS 2018 (kg)	0.85		41
BQBRS12 Site	NIS 2019 k(g)	2.26	gVOL (m^3)	2.61
BQBRS13 Site	NIS 2019 (kg)	1.56		3.02
BQBRS12 Site	CNM (kg)	18311	gYE (kg/m3)	1.43
BQBRS13 Site	CNM (kg)			2.21
BQBRS12 Site	TKR (%)	41	Selection index ranking	-
BQBRS13 Site	TKR (%)	41		

Trait description:

NIS 2017= Nut in shell yield in 2017

NIS 2018= Nut in shell yield in 2018

NIS 2019= Nut in shell yield in 2019

CNM= cumulative nut in shell yield over three years (2017-2019)

TKR= Total kernel recovery

HGT= Height of the tree

gCNM= clonal value for nut in shell yield over three years (2015-2017)

gTKR= clonal value for total kernel recovery

gHGT= clonal value for height of the tree

gYE= clonal value for yield efficiency

UQM54

Selected for RVT4

Female Parent	A4
Male Parent	791

Raw Data		Genetic data		
Traits	**BQBRS13 data**	**Traits**	**Sites**	**Predicted value**
Yield 2017 (kg)	3.48	gCNM (kg)	BQBRS12 Site	11.46
Yield 2018 (kg)	5.93		BQBRS13 Site	5.87
Yield 2019 (kg)	8.89	gTKR (%)	BQBRS12 Site	39
cnm (kg)	18.3		BQBRS13 Site	40
tkr (%)	41	gVOL (m^3)	BQBRS12 Site	3.59
			BQBRS13 Site	3.21
		gYE (kg/m3)	BQBRS12 Site	3.19
			BQBRS13 Site	1.83
		Selection index ranking	BQBRS12 Site	1

UQM55

Selected for RVT4

Female Parent	849
Male Parent	A4

Raw Data		Genetic data		
Traits	**BQBRS13 data**	**Traits**	**Sites**	**Predicted value**
Yield 2017 (kg)	2.03	gCNM (kg)	BQBRS12 Site	5.00
Yield 2018 (kg)	1.17		BQBRS13 Site	6.12
Yield 2019 (kg)	3.16	gTKR (%)	BQBRS12 Site	43
cnm (kg)	6.36		BQBRS13 Site	44
tkr (%)	46	gVOL (m^3)	BQBRS12 Site	3.26
			BQBRS13 Site	3.32
		gYE (kg/m3)	BQBRS12 Site	1.54
			BQBRS13 Site	1.84
		Selection index ranking	BQBRS12 Site	2

UQM56

Selected for RVT4

Female Parent	BQBRS03-11-6
Male Parent	A268

Raw Data		Genetic data		
Traits	**BQBRS13 data**	**Traits**	**Sites**	**Predicted value**
Yield 2017 (kg)	3.41	gCNM (kg)	BQBRS12 Site	2.17
Yield 2018 (kg)	5.90		BQBRS13 Site	10.27
Yield 2019 (kg)	11.36	gTKR (%)	BQBRS12 Site	39
cnm (kg)	20.66		BQBRS13 Site	40
tkr (%)	43	gVOL (m^3)	BQBRS12 Site	2.81
			BQBRS13 Site	3.11
		gYE (kg/m3)	BQBRS12 Site	0.77
			BQBRS13 Site	3.30
		Selection index ranking	BQBRS13 Site	2

UQM57

Selected for RVT4

Female Parent	BQBRS03-11-6
Male Parent	A268

Raw Data		Genetic data		
Traits	**BQBRS13 data**	**Traits**	**Sites**	**Predicted value**
Yield 2017 (kg)	5.98	gCNM (kg)	BQBRS12 Site	2.06
Yield 2018 (kg)	6.85		BQBRS13 Site	12.15
Yield 2019 (kg)	14.19	gTKR (%)	BQBRS12 Site	39
cnm (kg)	27.01		BQBRS13 Site	39
tkr (%)	46	gVOL (m^3)	BQBRS12 Site	2.88
			BQBRS13 Site	3.22
		gYE (kg/m3)	BQBRS12 Site	0.71
			BQBRS13 Site	3.77
		Selection index ranking	BQBRS13 Site	1

UQM58

Selected for RVT4

Female Parent	849
Male Parent	791

Raw Data		Genetic data		
Traits	**BQBRS13 data**	**Traits**	**Sites**	**Predicted value**
Yield 2017 (kg)	4.05	gCNM (kg)	BQBRS12 Site	3.50
Yield 2018 (kg)	4.76		BQBRS13 Site	10.94
Yield 2019 (kg)	8.25	gTKR (%)	BQBRS12 Site	39
cnm (kg)	17.05		BQBRS13 Site	39
tkr (%)	39	gVOL (m^3)	BQBRS12 Site	3.24
			BQBRS13 Site	3.46
		gYE (kg/m3)	BQBRS12 Site	1.08
			BQBRS13 Site	3.16
		Selection index ranking	BQBRS13 Site	14

Appendix 1.3.2: Variability of initial and final nut setting in macadamia superior selections through different pollination methods.

Synopsis:

Selection for yield in Macadamia is a major component of the Australian breeding program because of its high economic weighting. Nut setting on a tree can be related to total yield and crop efficiency. Pollination success initially determines the nut set and consequently the yield of a tree. The method of pollination may affect the initial nut setting (INS) and final nut setting (FNS). We aim to analyse the variability of INS and FNS based on pollination methods in superior selection macadamia genotypes bred by the Australian Macadamia breeding program. Tested Genotypes are currently involved in a regional variety trial across northern New South Wales and South East Queensland, Australia. Pollinations took place in a high density Macadamia orchard containing 12 superior selection genotypes. Each genotype was pollinated using three methods; autogamous pollination, supplementary cross pollination by hand and natural open pollination. INS was recorded 65 days post pollination and FNS 180 days post pollination on 15 racemes per tree with 3 reps for each genotype. Pollination methods varied for both INS and FNS. The most efficient pollination method across genotypes was supplementary cross pollination. Autogamous pollination had low INS and lower FNS and the highest mean abscission rate (83.7%) due to the low rate of self-compatibility identified within the Proteaceae family. Because Macadamia is predominantly an outcrossing species, open pollination had lower variability between INS and FNS compared to the autogamous pollination method. Supplementary pollination also had the highest INS and FNS of all the pollination methods. Broad sense heritability analysis was used to estimate the heritability of INS and FNS per pollination method. (INS H2 INS=0.858-0.862, FNS H2=0.762-0846). Nut setting could be utilised for phenotypic selection of potential high yielding candidate Macadamia cultivars.

Appendix 1.4: Summary of chapters from Katie O'Connor's PhD thesis: Selection strategies to improve yield in macadamia using component traits and genomics

Appendix 1.4.1: Population structure, genetic diversity and linkage disequilibrium in a macadamia breeding population using SNP and silicoDArT markers

Introduction

Genetics and genomics studies now commonly use single nucleotide polymorphisms (SNPs) which are ubiquitous across the genome and can be discovered with ultra-high-throughput and low cost (Gupta et al., 2008). It is important to quantify the level of genetic diversity amongst modern crops to inform future breeding efforts (Glaszmann et al., 2010; Tanksley and McCouch, 1997). The genetic diversity and structure across the whole genome has not yet been measured for a large population representative of the macadamia breeding population. Diversity Arrays Technology Pty Ltd (DArT) can provide both bi-allelic codominant DArTseq-based SNPs as well as dominant binary (present vs. absent) silicoDArT markers, and does not require a reference genome (Kilian et al., 2003).

The present study is the first to analyse the genetic diversity of a large group of macadamia full-sib progenies and their parents, representing a breeding program, using SNP and silicoDArT markers. The objectives of this study were to: (i) determine marker locations on genome assembly scaffolds and calculate the extent of LD, (ii) determine the level of genetic diversity within and among a subset of families and parents in the Australian macadamia breeding program, (iii) analyse the extent of population structure, to inform future genomics studies, and (iv) determine if heterozygosity influences performance of nut yield.

Methods

This study was undertaken on a subset of a population that was part of the of the Australian macadamia breeding program's first generation population (Hardner et al., 2009; Topp et al., 2016a). The larger population comprised of a total of 1,961 progeny seedlings from 141 families between crosses of 47 parents designed to maximise diversity. These seedlings were planted between 1999–2003 in south-east Queensland and north-east New South Wales at nine sites (Hardner et al., 2009; Topp et al., 2016a). Thirty-two families were chosen as the subset for this study based on the family size being approximately ten progeny per family, giving a total of 295 seedling progeny. The families were from crosses between 29 commercially available parents (reciprocal crosses combined); all with at least one parent involved in another cross so there were no isolated families. Within each family, five low-yielding and five high-yielding offspring were chosen where available to ensure a range of phenotypes across families. The trees were at four orchard sites in south-eastern Queensland: two in the Bundaberg region (Alloway, AL, and Hinkler Park, HP) and two in the Gympie region (East Gympie, EG, and Amamoor, AM; Figure 2-1).

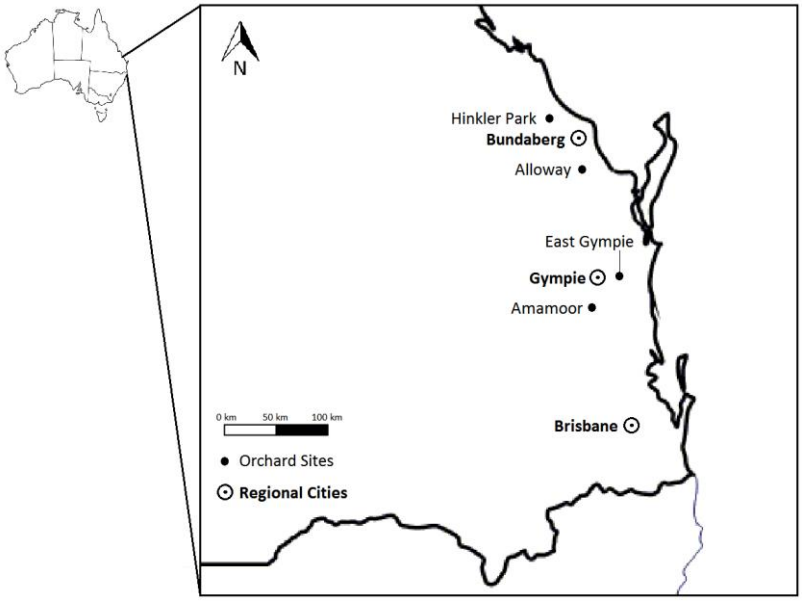


Figure 2-1: Locations of orchard sites and regional cities in south-east Queensland, Australia

Leaf samples from each tree were processed by Diversity Arrays Technology Pty Ltd (DArT, Canberra, Australia). DArT Pty Ltd's proprietary SNP and silicoDArT marker calling algorithms (DArTsoft14) were used. Imputation was performed on both SNP and silicoDArT markers through DArT's KDCompute Optimal Imputation plugin. Quality control was applied to both types of markers using pre-imputation read depth, call rate, one ratio, polymorphic information content (PIC), and a test of Mendelian inheritance on a subset of families. A v2 genome assembly for M. integrifolia is available as a collection of 4,098 scaffolds. The tentative locations of the quality-filtered markers on these genome scaffolds were found using the NCBI stand-alone BLAST+ application (Camacho et al., 2009). LD was calculated using the r2 parameter between pairs of SNPs within the same scaffold using Plink v1.9 software (Purcell et al., 2007).

Allelic frequencies were calculated in GenAlEx v6.5 (Peakall and Smouse, 2012). Diversity parameters included mean number of alleles per locus (A), number of effective alleles per locus (Ae), observed and expected heterozygosity (Ho and He), and percentage of polymorphic loci (%P) for each family. Analyses were conducted to determine if there was a relationship between genetic diversity and performance for nut yield and other traits. Principal coordinates analysis (PCoA), unweighted neighbour-joining dendrogram and STRUCTURE analysis were performed using SNPs to identify relationships and admixture within and among progeny families.

Results and discussion

After quality control and filtering, 4,113 SNPs and 16,171 silicoDArTs across 295 progeny and 29 parents remained for genetic analyses. A total of 3,700 SNPs (90%) mapped to 1,411 genome scaffolds (34%). Most SNPs were present at only one location (2,846, 80%) or mapped to two (13%) locations; the rest mapped to multiple genome scaffolds. One SNP (s3709) mapped to 119 scaffolds, with another (s3710) mapping to 51 scaffolds.

Genetic diversity varied slightly between progeny and parents (Table 2-4). For progeny, higher number of alleles per locus (A), number of effective alleles (Ae), expected heterozygosity (HE) and percentage of polymorphic loci (%P) were observed than for parents. Fewer heterozygotes were observed for both parents and progeny (HO = 0.135 and

0.124, respectively) than were expected (HE = 0.250 and 0.255, respectively; Table 2-4). Genetic diversity was higher for all measures for progeny from hybrid parents than progeny from *M. integrifolia* parents (A = 1.997, 1.804, and %P = 99.66%, 80.43%, respectively). There was virtually no correlation between heterozygosity and yield, kernel recovery or tree size traits (r = 0.05 to 0.22).

In the principal coordinates analysis (PCoA), there are five visible clusters amongst the progeny (circled; Figure 2-6a). Full-sib families that share parents clustered together in the PCoA and dendrogram (Figure 2-6b). Generally, progeny from hybrid parents (bolded) separated from progeny from *M. integrifolia* parents, both in the PCoA and dendrogram. The long dendrogram branches of progeny from hybrid parents, for example 'D4' x '695' reflects the amount of evolutionary change (Pavlopoulos et al., 2010) compared with progeny from Hawaiian cultivar germplasm, suggesting that the genetic diversity of hybrids is greater than those with *M. integrifolia* ancestry. The three ancestry clusters shown in the STRUCTURE output may represent three different germplasm groups (Figure 2-6c): *M. integrifolia* HAES germplasm (orange), and two different hybrid groups. Some findings of the current study conflict with records and publications. Cultivar '772' appears to have a mixed ancestry, despite records suggesting it originated from a wild *M. integrifolia* population (Peace, 2005). 'Yonik' is a variety from Israel with recorded *M. integrifolia* ancestry; however, evidence from the STRUCTURE plot suggests that it may also be a hybrid. Variety 'NG35' was recorded to be of hybrid origin (Peace, 2005), though, the results of this study suggest that it is more likely to be *M. integrifolia* due to its progeny clustering separately from hybrids.

Table 2-4: Summary of genetic diversity measures averaged over 4,113 SNP markers, for the parent population, across all progeny, progeny from *M. integrifolia* parents, and progeny from hybrid parents. n, number of individuals; A, number of alleles; Ae, number of effective alleles; HO, observed heterozygosity; HE, expected heterozygosity; %P, percentage of polymorphic loci

		n	**A**	**A_e**	**H_O**	**H_E**	**%P**
Parents	Mean	29	1.984	1.396	0.135	0.250	98.42%
	SE		0.002	0.005	0.002	0.002	
All progeny	Mean	295	2.000	1.403	0.124	0.255	100%
	SE		0.000	0.005	0.002	0.002	
Hybrid progeny	Mean	176	1.997	1.447	0.131	0.278	99.66%
	SE		0.001	0.005	0.002	0.002	
***M. integrifolia* progeny**	Mean	119	1.804	1.303	0.115	0.189	80.43%
	SE		0.006	0.005	0.002	0.003	

In conclusion, this study used 4,113 SNP and 16,171 silicoDArT markers to analyse a large, representative subset of progeny of the Australian macadamia breeding population, as well as many cultivars as parents. High genetic variance was observed among progeny and among full-sib families, and the population seems to be structured by Hawaiian cultivar *M. integrifolia* germplasm separating from hybrid germplasm. Knowledge gained will be valuable for future studies using genetic markers in macadamia. A genome-wide association study regarding important yield traits will benefit from the number of markers available as well as awareness of population structure to avoid bias and spurious results.

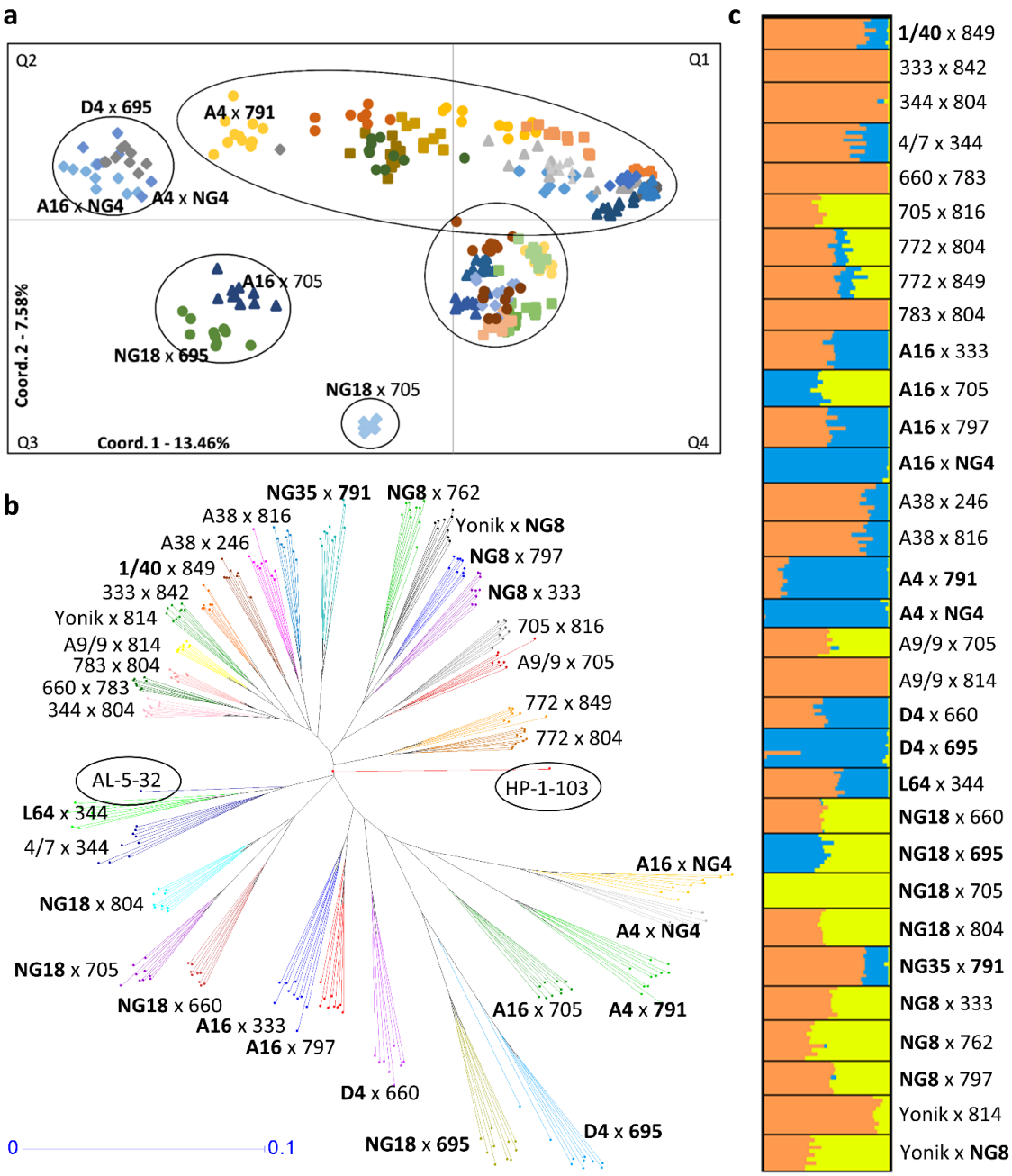


Figure 2-6: Population structure analysis of progeny families based on 4,113 SNP markers, with hybrid parent genotypes in bold. a Principal coordinates analysis with progeny coded according to full-sib families with five putative sub-clusters circled. b Unweighted neighbour-joining dendrogram based on genetic distance among progeny, with 100 bootstraps. Progeny are colour-coded and labelled according to their full-sib families. c Clustering of progeny among ancestries as calculated by STRUCTURE. Each horizontal line represents one genotype, and different colours represent partitioning of the genotype to each cluster, k = 3 clusters. Genotypes are grouped by full-sib family

Appendix 1.4.2: Genomic heritability, correlations, and selection efficiency of nut yield and component traits in a macadamia breeding population

Introduction

Selecting for high yield can be difficult due to the quantitative nature of the trait, low heritability, long juvenile period, and large tree size requiring large areas of land for evaluations. Breeders could indirectly select for complex traits, like yield, through the evaluation of component traits (Piepho, 1995; Simmonds, 1979; Sparnaaij and Bos, 1993). Component traits with high correlation with the target trait, high heritability, and those that are more easily measured than the complex target trait, are candidates for indirect selection (Falconer, 1989; Sparnaaij and Bos, 1993). Some component traits can be measured at an earlier stage in the life of the tree, which can lead to reduced cycle times, or traits can be measured more easily on a larger number of trees, meaning that selection intensity is increased.

Heritability of traits and genetic correlation with yield can be estimated using linear mixed model approaches, and these estimates can then be used to calculate the efficiency of indirect selection on the target trait through the direct selection of another trait (Falconer, 1989). The efficiency of selection depends on the ratio of the correlated response of the two traits to the direct response of the target trait. The aims of this study were to: (i) compare the phenotypic variances of nut, floral and growth component traits between progenies and families, (ii) estimate the genetic heritability of yield and component traits in the population, (iii) estimate genetic correlations among yield and component traits, and (iv) estimate efficiency of indirectly selecting for high yield through the selection of component traits.

Methods

This study uses the 295 progeny and their parents, as well as the 4,113 SNP marker data outlined in the chapter above. The progeny, as well as 18 of the 29 parents, were measured for yield and twelve yield component traits from August 2016 to July 2018 (Table 3-1).

Ten racemes per tree, and 20 good quality nuts per tree were measured for multiple component traits (Table 3-1). Nuts-in-shell were harvested by hand for each tree, as a measure of yield. Site AL was not harvested in 2017 due to an extreme weather event, whilst site EG was not harvested in 2018 due to management issues. Means of each trait were calculated for each individual for each yield component.

Traits that were not normally distributed were transformed to approximate normality of within-site residual distribution, and then scaled. Univariate and bivariate models were implemented using ASReml-R (Butler et al., 2009). Narrow-sense heritability on a single tree basis was estimated for each trait using univariate models. Pairwise bivariate models were used to estimate genetic correlations between yield and each of the component traits. Efficiency (E) of indirect selection of high yield using a component trait compared with direct selection of yield was calculated for each component trait using the heritability and genetic correlation estimates.

Results and discussion

Very low NW and KW were observed in family ‘NG18’ x ‘695’ (NW 4.34 g) and ‘NG8’ x ‘762’ (1.46 g KW), with an average of 7.09 g and 2.73 g, respectively. Average KR was 38.7% and ranged widely from 20.2% in ‘A38’ x ‘246’ to

55.6% in 'L64' x '344'. Previous calculations of the percentage of florets that set nuts (FSN) were largely based on broad estimates using the number nuts per raceme and flowers per tree, at 0.3% (Ito, 1980). Measuring techniques used here are presumed to be much more accurate, and phenotypes ranged from 0.4 to 7.2%, with a mean of 2.0.

Table 3-2: Summary of phenotypes for yield and yield component traits across all individuals and all sites: raw untransformed maximum, minimum, average, standard deviation (SD). The family of the tree (or cultivar, cv.) with the lowest and highest values is shown. Multiple indicates that there was more than one family or cultivar with that value. * denotes measured over two seasons at some/all sites

Code	Trait	Average	SD
ENF	Estimated number of florets per raceme	139	49
FSN (%)	Flowers that set nuts (NPR / ENF)	2.0	1.2
KR (%)	Kernel recovery (KW / NW)	38.7	0.05
KW (g)	Kernel weight	2.73	0.55
NPR	Number of nuts per rachis	2.6	1.4
NW (g)	Nut-in-shell weight	7.09	1.34
PD (mm)	Nut pedicel diameter	3.47	0.55
RDN (mm)	Rachis diameter at nut set	3.61	0.85
RL (cm)	Raceme length	12.0	3.9
RSN (%)*	Racemes surviving from flowering to nut set	25	24
TC (cm)	Trunk circumference	51	12
WK (%)	Whole kernels	64	17
Yield 2017 (g)*	Nut-in-shell yield	4,737	5,499
Yield 2018 (g)*		5,910	5,422

Estimates of heritability and genetic correlations between traits will inform future breeding efforts, regarding the ease of selecting for certain traits. Heritability ranged from 0.09 for RSN to 0.76 for KR, with yield being moderately low at 0.31 (Table 3-3). The high heritability for KR means that increasing the average KR in breeding populations (38.7% in this population) towards desirable levels (>40%) (Topp et al., 2019) is achievable through selection. Heritability of NPR was lower (0.17) than expected in the current study, based on the phenotypically-observed consistency of nuts per cluster within a tree. The estimate of narrow-sense heritability in this study for yield (h2 = 0.31) is similar to that previously estimated for NIS yield at age 7 years for progeny generated from a different set of parents, ranging from 0.35–0.46 across sites using pedigree data (Hardner, 2017). Very little G x E (site) variance was observed for most traits.

Estimated genetic correlations with yield varied among traits from -0.27 for KR to 0.99 for RSN, with an average of 0.31 (Table 3-3). The slight negative correlation between KR and yield (rg = -0.27) in the current study concurred with previous results (Hardner et al., 2002). The results of the current study imply that selecting for high KR may tend to lead to lower yields, and vice-versa, and may have implications for selection in the breeding program, since both high yield and high KR are selection priorities. Since yield in macadamia is generally based on NIS weight across the whole tree, the positive genetic correlations between yield and both NW (0.40) and KW (0.21) were expected, since heavier kernels and nuts will lead to heavier tree yields. Since NW and KW are both traits with intermediate optimums (O'Hare et al., 2004), selecting for large nuts to increase yield is not necessarily desirable, as current

machinery may be unable to process very large nuts. The aim to develop smaller trees with high productivity (Toft et al., 2019) may be difficult for macadamia, due to the strong positive correlation between TC and yield (0.72), which implies that larger trees produce more nuts.

Estimates of selection efficiency using component traits to indirectly select for high yield varied from 0.03 (WK) to 0.86 (TC). The negative genetic correlation between yield and KR resulted in -0.42 selection efficiency for that trait. None of the traits had a higher selection efficiency for indirect selection than direct selection for yield (1.00). The selection efficiencies calculated for TC and NW suggest they could be contenders for indirectly selecting for high yield, if the cost of assessing these traits was much lower than assessing yield, as neither were equal to or more efficient than directly selecting for yield. However, as previously mentioned, it is not in the interest of the breeding program to select for larger trees or nut sizes. As such, the results here indicate that none of the studied component traits are suitable for use in indirectly selecting for high yield through correlated response to selection.

Appendix 1.4.3: Genome-wide association studies for yield component traits in a macadamia breeding population

Introduction

The use of genomics in plant breeding is expanding (Grattapaglia and Resende, 2011; Iwata et al., 2016; Khan and Korban, 2012), including employing genome-wide association studies to identify molecular markers associated with important traits, and genomic selection for complex traits. A common approach is using genome-wide association studies (GWAS): each marker (typically single nucleotide polymorphism, SNP) is analysed individually to detect evidence of marker-trait associations (Khan and Korban, 2012). This method relies on linkage disequilibrium (LD) between markers and causal polymorphisms. Findings of GWAS can be followed by marker-assisted selection (MAS) if a reasonable proportion of trait genetic variation is explained by the significant markers. In MAS, candidates are screened for target markers, their phenotypes are predicted based on allelic states, and selections can be made based on these predictions (Collard et al., 2005; Tester and Langridge, 2010).

GWAS coupled with MAS at these specific loci is a feasible option for improving yield component traits in macadamia; hence, we aim to investigate this option in the Australian macadamia breeding program. Target traits for GWAS and potential MAS in macadamia include commercially important traits, including nut and flowering characteristics, as well as tree size. The aims of this study were to: (i) perform GWAS to identify markers significantly associated with yield component traits, and (ii) determine the location of significant markers on genome scaffolds, and calculate LD between significant marker pairs.

Methods

This study involves study material, genotypic data and phenotypic data collected as in previous chapters. A genomic relationship matrix (GRM) was constructed following methods of (VanRaden, 2008). Preliminary analysis was performed using ASReml (Gilmour et al., 2009) in R for each trait, with fixed effects (site, block within site, tree type = grafted parent or seedling progeny). The significance of fixed effects was determined using the Wald statistic, and insignificant fixed effects were removed (individualised for each trait). Log likelihoods of models both including and excluding G x E as a random term were compared via a chi-square test to determine if the models were statistically different; the G x E term was excluded for a trait if the models were not statistically different.

Association analysis was performed for each trait using ASReml (Gilmour et al., 2009) in R, using a mixed model by including each SNP marker one at a time as a fixed effect. QQ (quantile-quantile) plots were constructed for each trait to evaluate whether population structure had been accurately accounted for in the model. A false discovery rate (FDR < 0.05) was calculated for each trait with the BH method (Benjamini and Hochberg, 1995) using the p.adjust function in R. Multiple regression was performed for traits with multiple significant associations, where significant markers were included as fixed effects, to determine if any SNPs were linked. Markers that were no longer significant after regression were deemed to be detecting the same QTL as one of the significant markers, and as such were considered redundant. The proportion of additive genetic variance explained by significant SNPs for each trait was calculated using allelic frequencies, genetic variance and marker effects. Locations of significant SNPs (FDR > 0.05) on the most recent macadamia genome scaffolds (v2; 4,098 scaffolds. LD was measured using the r2 parameter between all pairwise significant SNPs from the current study using Plink v1.9 (Purcell et al., 2007), with scaffolds as pseudo-chromosomes.

Results and discussion

Log-transformed (log10(x)) observations for NW, KW and NPR, as well as square root transformed observations for RSN appeared more normally distributed than raw observations (Figure 4-1). Yield (2017 and 2018) was not normally distributed, and neither log (log10(x), ln) nor square root transformations led to more normally distributed data, even for individual sites. This indicated that GWAS is not appropriate for yield, and association analysis was not performed for this trait.

The GRM appeared to have effectively accounted for population structure in all traits except for TC, as no more associations than expected by chance were observed at low levels of significance in the QQ plots (Figure 4-2) (Gondro et al., 2013). One explanation for divergence from the null hypothesis (more associations detected than expected) at high p-values, as observed for TC, is polygenicity: many loci of small effect contributing to variation in the trait (Yang et al., 2011).

GWAS identified seven SNP markers significantly (FDR < 0.05) associated with NW, four with WK, and 44 with TC (Figure 4-2; Table 4-3). After multiple regression, where significant SNPs were treated as fixed effects, some markers were no longer significantly associated with some traits. Only SNP s2204 remained significantly associated with NW, whilst SNP s2607 was no longer a significant association for WK (Table 4-3). Multiple regression for the four markers significantly associated with WK found that the unmapped SNP s2607 was redundant. Furthermore, the LD between the two mapped markers for WK was low, at r2 = 0.082, suggesting that they were unlinked and representing two separate causal variants. For TC, 14 of the 44 significant markers were non-redundant, suggesting that there may be 14 QTLs controlling this trait. Linkage disequilibrium between significant associations within each trait varied from r2 = 0.082 between two markers for WK, to 1.000 between six SNPs for NW (Table 4-3).

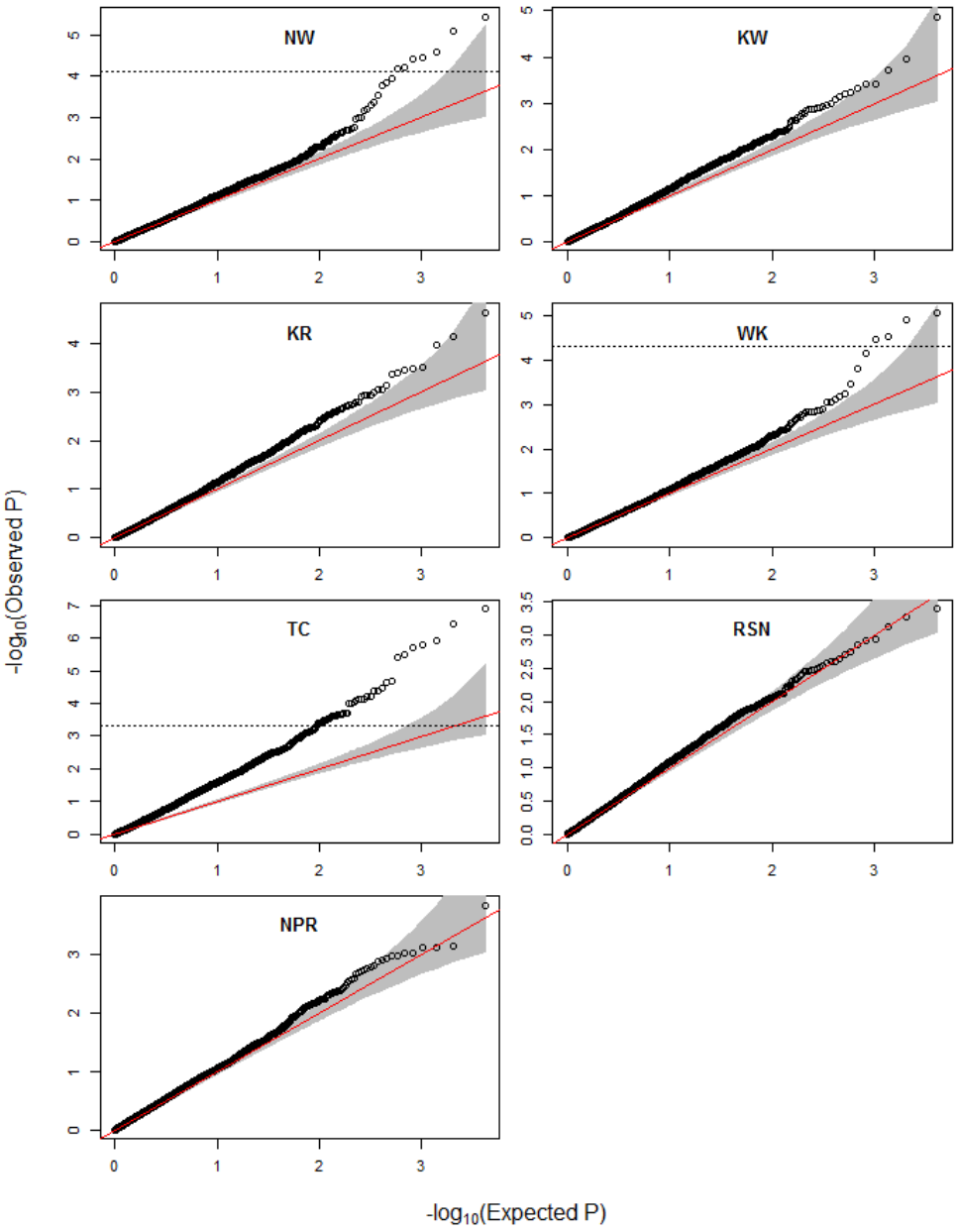


Figure 4-2: QQ plots showing expected significance levels against observed significance for 4,113 SNPs for yield component traits. Red diagonal lines indicate the null hypothesis, where observed and expected p-values would sit if there were no associations. Dashed horizontal lines indicate FDR = 0.05, SNP markers above which were deemed significantly associated with the trait. Shaded area indicates 95% confidence interval

Table 4-3: Summary of significant SNPs associated with yield component traits identified in GWAS. Only the ten most significant markers for TC are shown. MAF, minor allele frequency of the marker; p, significance of association; pMR, significance of association as determined by multiple regression with significant SNPs as fixed effects; NS, not

significant. - indicates marker was not mapped to scaffolds. a Scaffold in v2 genome assembly. b Did not pass FDR = 0.05 threshold

Trait	SNP	Scaffold[a]	Alleles	p	pMR
NW	s2204	scaffold926|size239084	A/G	3.68E-06	2.147e-05
	s4163	scaffold285|size451335	C/T	8.03E-06	NS
	s1434	scaffold_177	T/C	2.65E-05	NS
	s1643	scaffold44|size832018	A/C	3.46E-05	NS
	s1121	scaffold653|size305054	A/G	3.82E-05	NS
	s5182	-	A/T	6.29E-05	NS
	s2256	scaffold710|size289053	G/T	6.45E-05	NS
KW	s3540[b]	-	G/A	1.34E-05	
KR	s1707[b]	scaffold_72	C/T	2.37E-05	
WK	s0201	scaffold213|size509421	G/A	8.81E-06	4.10E-05
	s1917	-	A/G	1.23E-05	1.61E-02
	s2607	-	T/C	2.91E-05	NS
	s3239	scaffold361|size1112638	G/C	3.39E-05	5.68E-04
TC	s3169	-	T/C	1.29E-07	2.92E-03
	s1053	scaffold597|size318270	G/A	3.82E-07	9.16E-03
	s3616	scaffold364|size402398	T/C	1.15E-06	4.03E-02
	s2631	scaffold1151|size292196	G/C	1.67E-06	NS
	s3332	scaffold1221|size537814	T/C	1.97E-06	1.65E-03
	s4480	scaffold_221	A/T	3.20E-06	2.67E-02
	s3406	-	G/A	3.82E-06	NS
	s2500	-	G/A	2.02E-05	3.46E-02
	s0011	-	G/C	2.20E-05	6.07E-02
	s3405	-	G/C	3.40E-05	NS

The average phenotypes of NW at SNP s2204 for AA were small (7.03 g, n = 309, SD = 1.29), and large for GG (9.54 g, n = 6, SD = 1.73) (Figure 4-3). Similarly, the average values of WK for AA were high (78.0%, n = 5, SD = 11.0), and low for GG (62.3%, n = 265, SD = 16.8). As a very simple (overestimated) demonstration for how MAS could be used, breeders could genotype seedling progeny from their very first leaves at these key markers, and select for AG heterozygotes at SNP s2204 for intermediate nuts, and AA genotype at SNP s0201 for a high percentage of whole kernels.

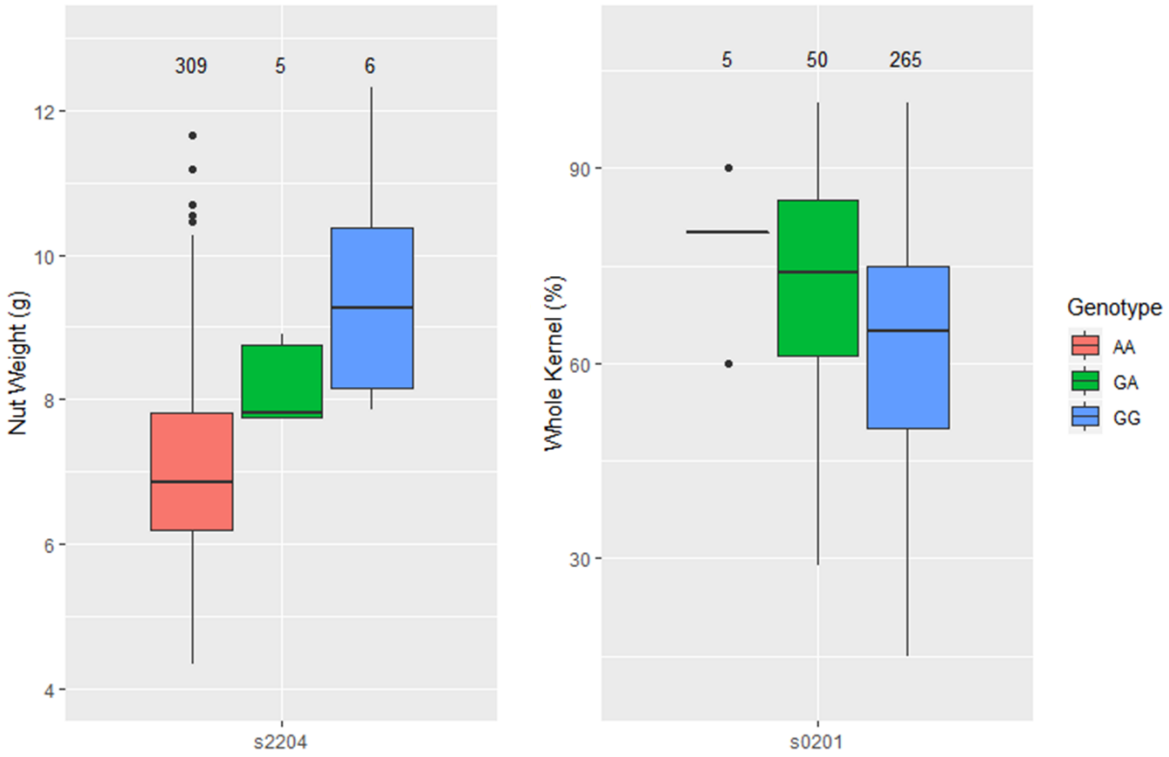


Figure 4-3: Boxplots showing distribution of raw, untransformed phenotypes across three genotypic states for markers s2204 and s0201 significantly associated with nut weight and percentage of whole kernel, respectively. Numbers above each box represent the number of trees with that genotype for that marker.

A separate population should be studied to determine if QTLs detected are the same as those detected here, or are new associations. Further studies should incorporate larger population sizes, to ensure that significant associations are accurate and applicable to a wider breeding population. When a more complete reference genome is assembled, the location of these markers can be estimated, and LD between markers more accurately estimated with population structure and cryptic relatedness taken into account. Other traits that could be analysed include self-fertility, and resistance to diseases that affect nut yield, including husk spot and phytophthora.

In conclusion, the findings of this study have important implications for macadamia breeding, but it also highlights the difficulties of employing GWAS in heterozygous populations with rapid LD decay. By coupling validated marker-trait associations detected through GWAS with MAS, genetic gain could be increased by decreasing the selection time for economically important nut characteristics and other yield component traits.

Appendix 1.4.4: Genomic selection for nut yield and yield stability, and a comparison of selection strategies in the Australian industry

macadamia breeding program

Introduction

Although yield is the main trait of focus when selecting new macadamia varieties, it is expensive and difficult to assess in breeding programs. Due to the crop's long juvenile stage, as well as the need to assess yield over several years to increase the accuracy of prediction of long term yield, traditional macadamia breeding has a selection cycle of almost a quarter of a century (22 years) (Hardner et al., 2009; Topp et al., 2016). Alternative selection strategies are sought to shorten the selection cycle and increase genetic gain.

Genomic selection utilises genome-wide markers to predict genomic estimated breeding values (GEBVs) of individuals, after which the best performers are selected (Meuwissen et al., 2001; Viana et al., 2016). GS uses a training or reference population of individuals with known genotypes and phenotypes to construct a model of each marker's effect on the trait. The model is then applied to a population with known genotypes but unknown phenotypes to predict their genomic estimated breeding value (GEBV) for a particular trait. First, the model is tested with a measured population; the accuracy of prediction is determined by how closely the predicted values reflect the observed phenotypes (correlation). The GEBV can be predicted for individuals at the seedling stage, using only genotypic data; thus, accelerating breeding cycles, enabling early selection for elite individuals and thereby increasing the gain per unit time (Denis and Bouvet, 2013; Desta and Ortiz, 2014; Jannink et al., 2010; Meuwissen et al., 2001).

We hypothesise that using GS in the macadamia breeding program will lead to greater genetic gains than phenotypic- and pedigree-based selection, due to a substantial reduction in generation length. This study aims to: (i) determine the accuracy of GBLUP (genomic best linear unbiased prediction) methods in predicting GEBV for nut yield and yield stability across years in macadamia; and (ii) identify strategies in which GS can be employed to increase genetic gain in macadamia breeding programs. This research is the first study to utilise molecular marker technology for GS in macadamia, and to our knowledge, the first to use GS to predict yield stability over consecutive years for a fruit or nut tree crop.

Methods

This study involves the progeny population across four sites in Queensland, and SNP genotypes described above. Yield data from young trees only (aged 5 to 8 years) and yield data from mature trees only (2017 and 2018 harvests, aged 14–17 years) were used in analyses. GBLUP models were used to calculate GEBVs for each tree using ASReml-R.

Initially, phenotypes were analysed by excluding genetic data to obtain phenotypes corrected for fixed effects (solutions, or corrected phenotypes). Terms in the model included site, year, type (seedling or parent), number of neighbours within row, tree age and interactions as fixed effects, and genotype by site as a random effect. Mean yield across multiple years and cumulative yield (summed across years) were compared. Yield stability, was calculated as the standard deviation of corrected phenotypes over multiple years. Estimates of genomic narrow-sense genomic heritability (h2) for yield and yield stability were calculated.

In the next step, SNP data were included in analyses to model pedigree and relatedness using a genomic relationship matrix. The accuracy of the GEBVs from the models above were determined using five-fold cross-validation (CV). In turn, 20% of phenotypes were masked (set to missing) in a validation set, and data for the remaining 80% of individuals were used as a training set to train the model and predict the missing values. This process was repeated five times. Individuals were assigned to one of five groups using two grouping techniques: random and related family groups. For each CV, prediction accuracy (r) was calculated as the correlation between GEBVs (predicted

phenotypes, using SNP data) and corrected phenotypes (no genetic data or pedigree used) divided by the square root of the genomic narrow-sense heritability. Genetic gain (ΔG, grams/year) was calculated for traditional breeding and GS methods using selection intensity, heritability/accuracy, and generation length.

Results and discussion

Narrow-sense heritability for yield was 0.23, and for yield stability across years was 0.04. The highest yield prediction across all models was achieved when sites were analysed individually and then accuracies averaged across sites (Figure 5-3). Prediction accuracies were similar for young-tree data analysed with all sites together ($r = 0.57$, $p < 0.05$) and for phenotypes corrected for individual sites and then combined (0.58, $p < 0.05$) using random groupings. Calculating corrected phenotypes for individual years and then summing to obtain cumulative yield did not produce more accurate predictions than mean yield across years. Yield stability had high prediction accuracy for random groups (0.79, $p < 0.05$), and moderate for unrelated population predictions (0.28), though this was not significantly different from zero (Figure 5-4). Prediction accuracies for random groupings were consistently better than family groupings (predictions in unrelated populations). This is because with random groupings for CV, the training set includes full-sibs from the validation set (e.g. progeny from the same cross will be split across the training and validation sets), and so large blocks of chromosomes will be shared between the training and validation sets. Implementing GS in a population closely related to that which the model is based from would provide more accurate predictions of yield.

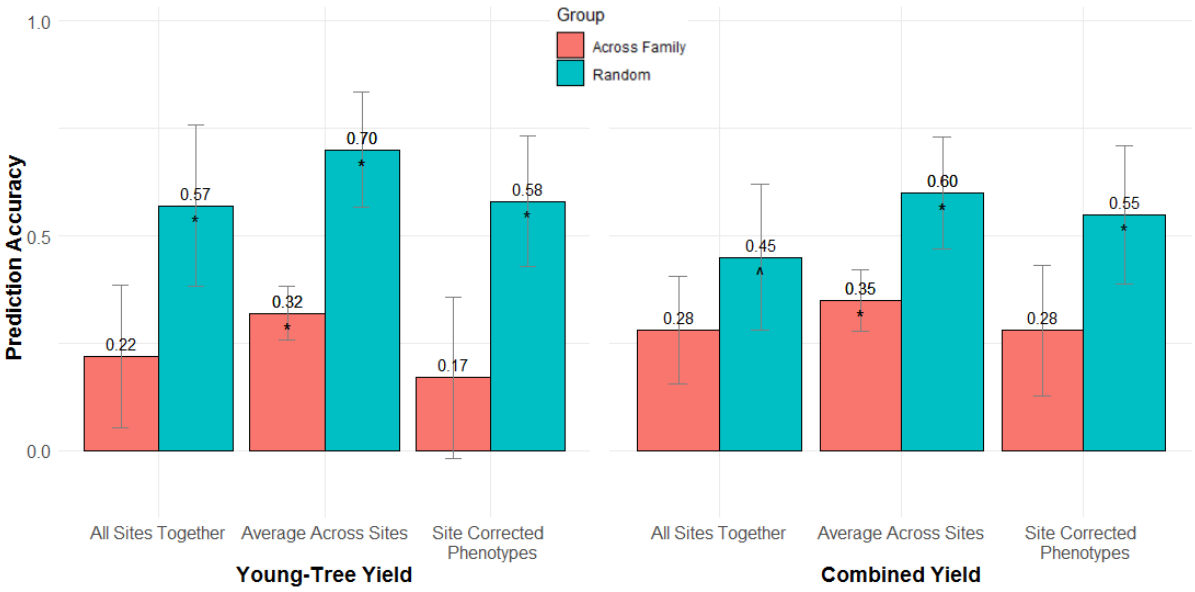


Figure 5-3: Mean prediction accuracy of yield across three methods: using phenotypes corrected with all sites analysed together (all sites together), an average accuracy across the four individual sites (average across sites), and using phenotypes corrected from individual sites and then combined (site corrected phenotypes). Accuracies are compared for two datasets: young-tree yield and combined young and mature-tree yield, with two cross-validation methods: randomly grouped individuals (predictions in related populations) and individuals grouped by family (predictions in unrelated populations). p-values indicate whether accuracies are significantly different to zero: * $p < 0.05$, ^ $p = 0.06$; blank, not significant. Error bars indicate standard error of correlations from five cross-validations.

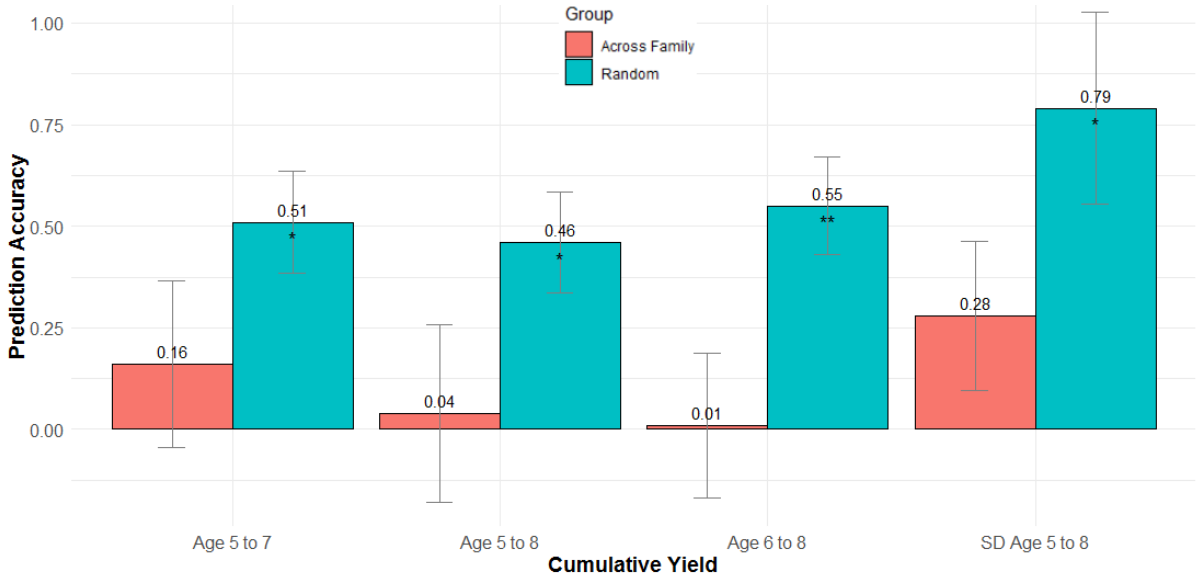


Figure 5-4: Prediction accuracy of cumulative yield and yield stability as a function of standard deviation (SD) of yield from age 5 to 8, using individual year GBLUPs across all sites together.

Genetic gain varied between GS models from 12 to 673 g/year, and was lower for 2.5% selection intensity than 1% (Table 5-3). Genetic gain was greatly influenced by the length of the breeding cycle (L); L for traditional breeding was eight years, and for GS it is four years, as elite individuals are identified from genetic markers using the first leaf, but cannot be used as parents until reproductive maturity (around the age of 4) (Hardner et al., 2009). Genetic gain using traditional breeding methods was 202 g/year for 1% selection intensity (Table 5-3).

Table 5 3 Genetic gain of yield and yield stability (in g/year) for each selection method and unrelated population or random cross-validation techniques. Genetic gain was calculated using accuracy of genomic selection (GS) model or square root of yield heritability for traditional breeding, standard deviation of corrected phenotypes (yield stability), generation length of four years for GS methods and eight years for traditional breeding, and 1% (i = 2.665) and 2.5% (i = 2.338) selection intensity, as per Equation 5-9.

Breeding method	**Genetic gain ΔG (g/year)***	
	p% = 1	**p% = 2.5**
Traditional Breeding	202	177
Family grouped (unrelated population) GS		
All Sites Raw Phenotypes	185	162
Cumulative Age 5 to 7	163	143
Cumulative Age 5 to 8	45	40
Cumulative Age 6 to 8	14	12
Yield stability (SD Age 5 to 8)	174	153
Randomly grouped (related population) GS		
All Sites Raw Phenotypes	480	421

Cumulative Age 5 to 7	534	468
Cumulative Age 5 to 8	482	423
Cumulative Age 6 to 8	673	590
Yield stability (SD Age 5 to 8)	488	428

* For cumulative yield, genetic gain is divided by the number of summed years

Genotyping seedlings using their first leaf after germination, to identify high-yielding individuals through GS, could halve the length of the seedling progeny trial. Then, elite trees could be crossed to produce the next generation as soon as they begin to flower, usually around the age of four. To reduce genotyping costs, delaying GS to deploy on a smaller population size may be a viable option. Seedlings could be grown out as per a traditional SPT, but only evaluated to age four, and precocious (early bearing) trees evaluated for KR. Breeders could pre-select precocious seedlings with high KR, genotype this reduced number of elite individuals, and then the highest-yielding trees could be selected through GS for evaluation in RVTs.

Increasing the size of a phenotyped and genotyped training population would increase the accuracy of yield prediction in macadamia. LD between markers and genes controlling target traits is essential for GS (Meuwissen et al., 2001). Increasing the number of markers used in GS may not necessarily achieve better accuracies, but increasing the density of markers across the genome could lead to increased prediction accuracies, as suggested by Calus et al. (2008), where models with r2 = 0.2 between markers were more accurate than models with fewer markers and lower densities. It is necessary to recalibrate the model after every few generations, as genetic variance explained by the markers will change, along with the allelic frequencies in the population (Goddard, 2009; Resende et al., 2012).

Future work employing GS to increase genetic gain in macadamia could investigate other economically important traits, such as tree size (trunk circumference), resistance to diseases, including husk spot and phytophthora (Drenth et al., 2009). Further work could also include multi-trait models, to investigate whether the inclusion of additional traits, such as trunk circumference and nut weight, increases the accuracy of yield prediction. The significant associations identified between traits and markers, as found in the GWAS chapter, could be incorporated into GS models. Different genomic prediction methods including BayesR and BayesB could be tested in the future to determine which are the most accurate in predictions. Finally, future GS analyses should involve more genetic markers across the genome. This may ensure that small-effect loci are captured, since LD in macadamia decays rapidly over short distances.

In conclusion, we have found moderate to high prediction accuracies applying GS models for yield and yield stability prediction in macadamia. Results from this study indicate that GS is a viable option to increase genetic gain in macadamia, though more research and resources are needed to increase the size of the training population, and phenotype and genotype these individuals to capture markers in LD with causal polymorphisms. This work could be combined with GWAS and MAS for key nut traits, and future work could also investigate other traits, multi-trait models and Bayesian models.

Appendix 2.1: Macadamia Rootstock Screening for High Performance: Genetic diversity, striking rate, early field growth and precocity

Mobashwer Alam, John Wilkie and Bruce Topp

Introduction

Rootstocks in tree crops like Macadamia play a vital role on the crop performance. As a part of a dual plant system, rootstocks translocate water and nutrients (Ferree and Carlson, 1987) to the above ground scion and thereby, regulate resource supply for growth and development of vegetative and reproductive parts. Having a strong tap root system with resistance to several biotic and abiotic factors, rootstocks increase adaptability to adverse environmental conditions. Studies in horticultural crops identified that rootstocks improve yield efficiency through reducing tree size and increasing precocity (Westwood, 1993). Till tody, very few investigations were made on macadamia rootstocks to increase production efficiency in Australia and limited to few genotypes only (Hardner and McConchie 2006; Hardner, 2004; and Neal et al., personal communication). As a part of "Transforming subtropical/tropical tree crop productivity" research project, this study designed on a diverse range of macadamia root stocks aiming to manage the vigour of the scion and increase production efficiency. In this report we presented the following aspects of this project:

- Graft success of macadamia seedling and cutting rootstocks.
- Establishment success in the field.
- Genetic diversity of the rootstocks,
- Early growth
- Precocity

Materials and Methods

Developing rootstock seedlings and cuttings

A total 30 genotypes were propagated for evaluation as rootstocks in this trial (Table 1). The genotypes consisted of 6 high performing rootstock cultivars; 3 elite cultivars with high breeding values for harvest index; 6 cultivars with high yield efficiency; 5 potential dwarf genotypes from Australian breeding program; 1 AVG resistant cultivar; 8 wild germplasm including 3 *Macadamia jansenii*, 3 *M. ternifolia* and 2 *M. tetraphylla*. Genotypes were propagated from seeds and cuttings during April to October 2014. Seedlings and cuttings were initially grown in the water controlled mist house and then transferred to the shed house for further development as detailed in Alam et al (2016).

Grafted tree development and management

Scions of HAES741 were whip-grafted on 20th June 2016 onto 245 seedlings and 188 cuttings Rootstocks of Macadamia. Initially two HAES741 cultivars, which were planted in December 1982 at the arboretum of Maroochy Research facility, Nambour, QLD, were cinctured in March 2016. On the same date of grafting, healthy and disease free grafting woods were collected from previously cinctured branches. Around 15-20 cm long scions were prepared by notifying the base as slanting cut and top as horizontal cut. Graft woods were sterilized with chlorinated water and kept wet by wrapping with water soaked towels. The top of the rootstocks were cut off at a point of approximately 25 cm above the soil and matched with the graft-woods of same thickness to help to get a good match between cambium layers of rootstock and scion. Sloping cut of about 30 mm long was made at the base of the scion and on the cut end of the rootstock using small wood plane. The wood plane was sterilized with alcohol at regular

Table 1. Number of progeny in each of the open-pollinated families from diversified origin of macadamia genotypes used in the precocity trial. OP = open pollinated, HVP= Hidden Valley Plantation, Beerwah Australia; HAES= Hawaiian Agricultural Experiment Station, Hawaii, USA; Aus Heritage = Australian Heritage Cultivars , Aus Elite= Elite selections from the Australian macadamia breeding program, and Wild jansenii= *Macadamia jansenii* wild germplasm.

Family	Source	Parentage	# Progeny
HAES246	HAES	*Macadamia integrifolia*	21
HAES344	HAES	*Macadamia integrifolia*	21
HAES788	HAES	*Macadamia integrifolia*	24
HAES791	HAES	*Macadamia integrifolia*	19
HAES814	HAES	*Macadamia integrifolia (?)*	21
A268	HVP	HAES344 OP	12
A376	HVP	-	21
A38	HVP	*Own Choice OP*	20
A4	HVP	Renown OP(x Own Choice)	19
A538	HVP	-	20
BAMAM02-6-3	Aus Elite	NG8xHAES762	21
BQBRS97-2-46	Aus Elite	HAES 246xA16	21
BQBRS97-6-16	Aus Elite	A16xHAES 814	21
BQBRS98-10-111	Aus Elite	HAES 246xA16	21
BQBRS98-10-93	Aus Elite	A16xHAES 781	11
BQBRS98-11-35	Aus Elite	HAES 849xDaddow	18
BQBRS98-11-80	Aus Elite	HAES 814xA16	5
BQBRS98-13-115	Aus Elite	HAES 842xDaddow	12
BQBRS98-14-25	Aus Elite	A16xHAES 814	18
BQBRS98-14-93	Aus Elite	DaddowxHAES 246	16
BQBRS98-15-37	Aus Elite	DaddowxA16	21
BQBRS98-16-41	Aus Elite	DaddowxA16	23
BQBRS98-6-73	Aus Elite	HAES 842xA16	23
BQBRS98-6-79	Aus Elite	A16xHAES 814	14
BQBRS98-7-109	Aus Elite	HAES 842xDaddow	18
BQBRS98-7-74	Aus Elite	DaddowxA4	14
BQBRS98-8-87	Aus Elite	HAES 816xA4	13
BQBRS98-9-72	Aus Elite	HAES 842xDaddow	21
M141	AUS Elite	-	20
D4	AUS Heritage	-	22
Daddow	AUS Heritage	-	21
Macadamia jansenii	Wild jansenii	*Macadamia jansenii*	12

Grand Total	584

interval to avoid any contamination at the grafting region. The cut regions of rootstocks and scions were clamped together using cloth pegs to help hold the scion in place and were wrapped with tape from bottom upwards to produce an overlapping pattern. The scion and tape was painted with grafting mastic to prevent it drying out. The grafted plants were then transferred to shed house providing 30% shade with 4x15 minutes sprinkling per day. Plants were fertilized with soluble native fertiliser ('Searles Flourish') at 7 days intervals and were checked every second/third day to remove any new buds the rootstocks. Initial graft success was noted when the scion wood produces a new shoot and final graft success was counted before planting trial. All the plants were taken out of the shade house three weeks before planting to ensure proper hardening of the plants. Plants were watered two times a day during the hardening period.

Planting rootstock trial

The rootstock trial was planted on 4th April 2017 in a previously uncultivated paddock at Maroochy research facility, Nambour QLD. The soil of the land was prepared two month before planting and fertilized with recommended doses of nutrients after soil testing. The experiment was planted in an incomplete block design considering all the spatial variation across 8 rows, 20 columns and 5 blocks. 12 buffer plants were planted in the middle of the trial, where there was a "cricket pitch". The trail was planted as a 2 plant plots of grafted and non-grafted plants from same genetic source. Non-grafted trees of the pairs were planted in a zig-zag to make a staggered design. The trial consisted of 42 grafted cuttings and 106 grafted seedlings. In addition to the grafted trees, 4 cuttings of 'HAES741' were also included in the trial to make a comparison on growth and performance with the grafted scion. Second tree of the plots having 'HAES741' was filled with buffer plants. Buffer plants were planted around the trial to void any edge effect. Growth parameters of grafted and non-grafted trees will be evaluated in first 3-4 years; non-grafted ones from each pairs will be removed afterwards. The buffer plants paired with 'HAES741' will also be removed.

Genetic diversity analysis

27 rootstocks were genotyped in a Diversity Array technology (DArT) platforms using 4174 DArTSeq based SNP markers (Kilian et al., 2012). Principal Coordinates Analysis (PCoA) and relationships based on marker data explain the genetic diversity among the genotypes. A genetic dissimilarity matrix was constructed using DARwin v. 6.0.13 (Perrier et al., 2003) software to identify the genetic relationships among the genotypes as illustrated in the Neighbour-Join dendrogram. Clades strength in the dendrogram was tested by 20,000 bootstrap analyses. GenAlEx v. 6.5 was used to perform PCoA, which was based on the standardized covariance of genetic distances calculated for the markers under study, using 999 permutations.

Phenotyping for early growth

We phenotyped the following growth characteristics in November 2017 and May 2018 (Table 2). We estimated the growth rate for all the parameters, which includes Total height increase (THI), canopy width increase (CWI), canopy depth increase (CDI), rootstock height increase (RHI), Rootstock trunk circumference increase (RCI), scion height increase (SHI), and scion trunk circumference increase (SCI).

We also phenotyped for flowering in August-September 2018 and 2019.

Table 2. Growth characteristics measured in the macadamia rootstock trial.

Traits of study	Units	Measurement period		
		Nov-17	May-18	May-19
Total height (THT)	cm	√	√	√
Canopy width along(CWA)	cm	√	√	√
Canopy depth across (CDA)	cm	√	√	√
Rootstock Height (RHT)	cm	√	√	√
Rootstock Trunk circumference(RTC)	cm	√	√	√
Scion height (SHT)	cm	√	√	√
Scion trunk circumference (STC)	cm	√	√	√

Data analysis

Data on graft success, growth data were analysed using REML mixed model in Genstat19 (Payne, 2000) platform.

Results and discussion

Genetic diversity

A dendogram (Figure 1) was constructed using DARwin 6.0.13 to elucidate the genetic relationship among the macadamia rootstocks. A total of 4174 DArTseq based SNP markers were used to produce the dendogram. Genetic dissimilarity between pairs of rootstocks were estimated and was varied from 0.31 to 0.29 with an average of 0.18. Genetic dissimilarity was smallest among the accessions of Macadamia jansenii (M.jan1, M.jan2 and M. Jan3) and peaked between rootstock pairs 'A4' and "M.jan2", although the accessions of all *Macadamia jansenii* and *Macadamia ternifolia* showed greatest dissimilarity with all existing cultivars and elite genotypes used in this study.

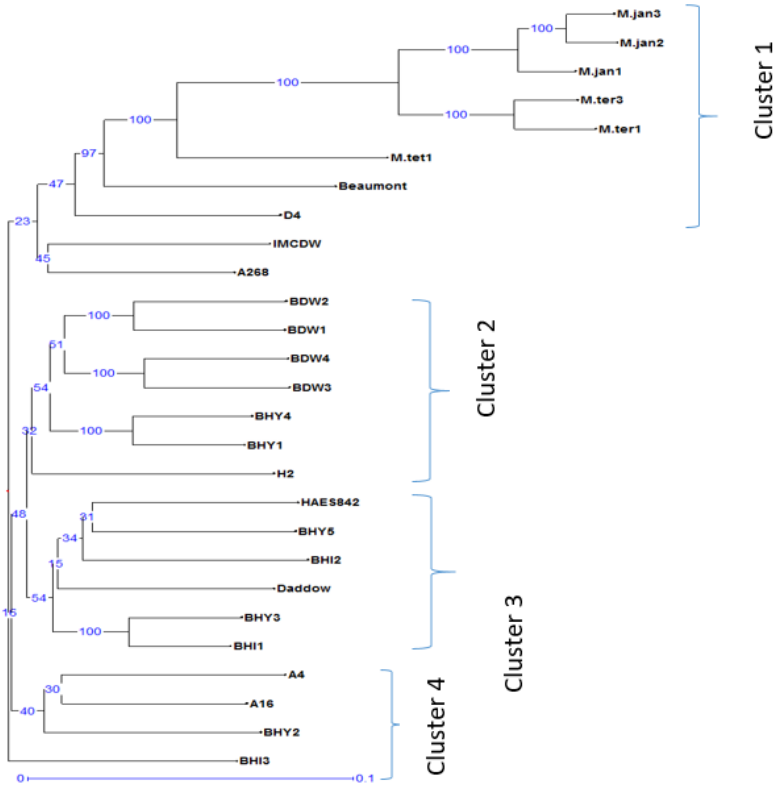


Figure 1. A dendogram showing genetic relationships among the macadamia rootstocks using 4174 DArTSeq based SNP markers.

In the dendogram, four main differentiating clusters of rootstocks were identified. First cluster contains all the wild germplasms. Cultivars “Beaumont”, ‘D4’, “IMCDW” and ‘A268’ also formed cluster 1 and indicated close relationship with *M. tetraphylla* (M.tet1). Four dwarf rootstocks (“BDW1”, “BDW2”, “BDW3” and “BDW4”) showed very close genetic relationships and formed the second cluster with “BHY1” and “BHY4”. Existing high performing rootstock ‘HAES842’ and AVG resistant cultivar “Daddow” were clustered with Elite cultivars “BHI1”, “BHI2”, “BHY3” and “BHY5”. The fourth cluster includes high performing macadamia rootstocks ‘A4’ and ‘A16’, and elite breeding lines “BHI3” and “BHY2”.

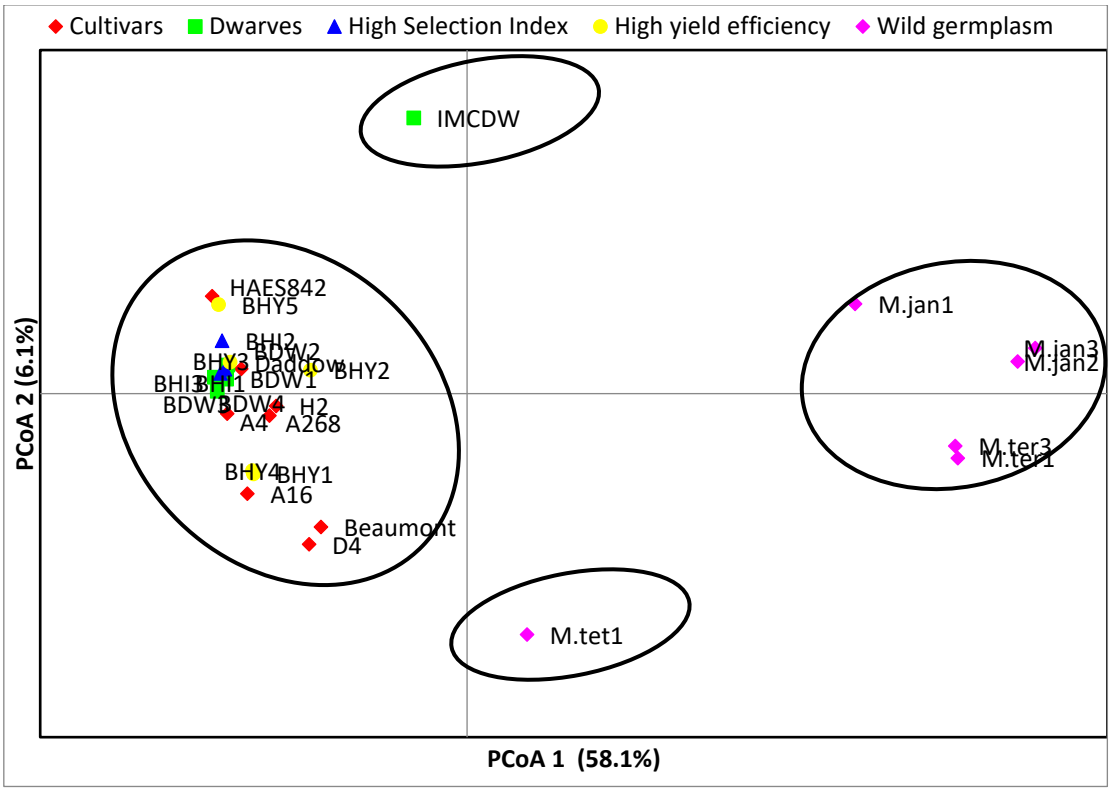
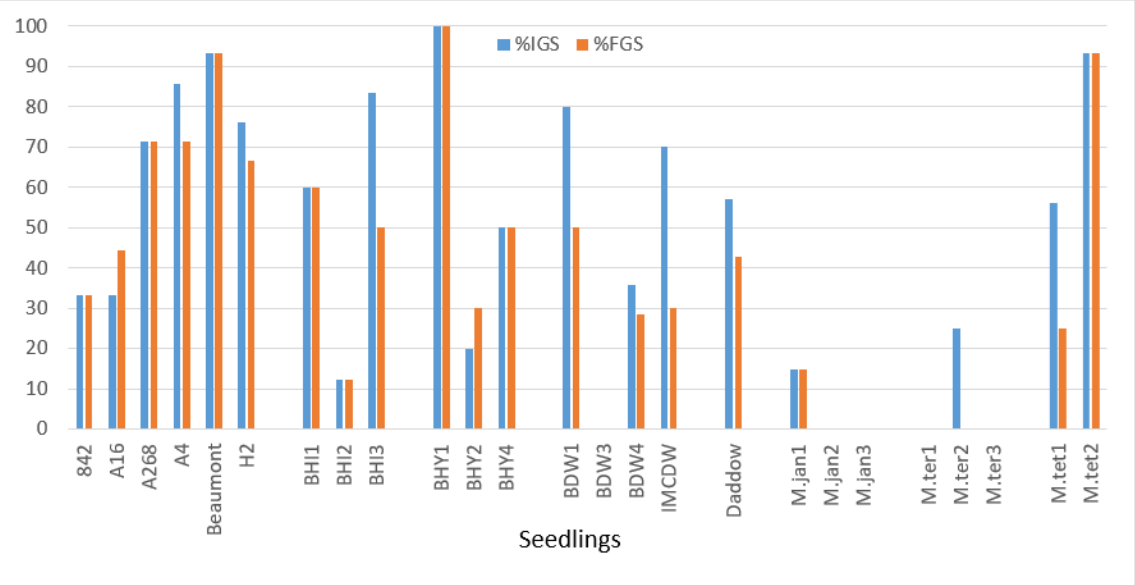


Figure 2. Principal Co-ordinate analysis of macadamia rootstocks using 4174 DArTSeq based SNP markers.

Principal coordinate analysis (PCoA) was performed through similarity matrix. First two principal components generated two distinct axes explaining 58.1% and 6.1% of the total variance respectively (Figure 2). PCoA identified four distinct groups with two group having single representative only. All the commercial cultivars, elites for high selection index and high performance, and dwarves (Except IMCDW) formed one group. Accessions from wild *M. jansenii* and *M. ternifolia* formed a separate group. IMCDW and M.tet1 were distinctly separated from both groups. Results thus clearly identified a wide range of diversity in the rootstock trial.

Graft success

An existing commercial cultivar 'HAES741' was grafted onto a total of 245 seedlings and 145 cutting rootstocks. There was significant variation in graft success of macadamia rootstock genotypes and methods of propagation (data not shown). The range of graft success varied from 0% to 100% in seedlings and 0% to 89% in cuttings (Figure 3). Over all, macadamia seedlings had greater graft success than that of cuttings. Variation between initial and final graft success was greater in cuttings than that of seedlings. Mostly dwarf genotypes and wild germplasm showed significant death of initially shooted grafted seedlings. While in cuttings, most of the genotypes had significant death of initially shooted grafted trees as observed by the reduction of graft success at final evaluation (%FGS).



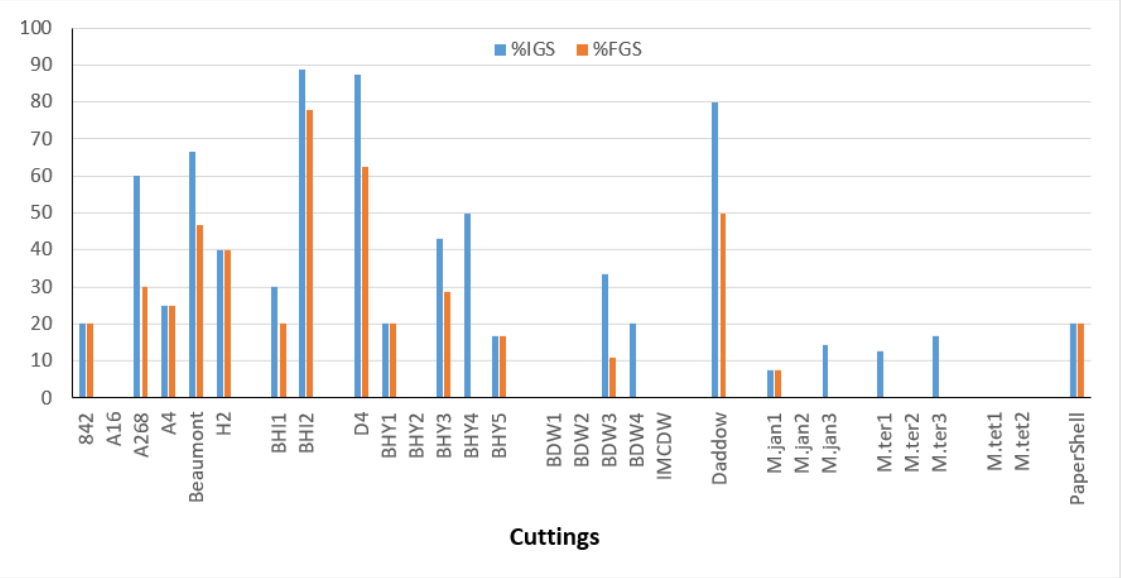


Figure 3. Initial and final graft success rate in macadamia seedling and cutting rootstocks.

In both seedlings and cuttings, Beaumont had greater graft success than other cultivars. Seedlings of "BHY1" and "M.tet2" were most compatible rootstocks with 'HAES741' due to their high rate of graft success in comparison with other seedling genotypes. Where as in cuttings, "BHI2", 'D4' and "Daddow" were on top for IGS and FGS. We previously reported that all these five genotypes had very high early vigour in terms of stem diameter. It is also to be noted that "M.tet2" was not tested cutting rootstock.

Among dwarves, BDW1 seedlings had higher rate of IGS (~80%) than "H2", whereas all the cuttings showed very low (0 to 34% for IGS and 0-12% for FGS) graft success. But FGS of "BDW1" seedlings was reduced to ~50%. Similarly, "IMCDW" had greater IGS (~70%) and lower FGS (~25%). Graft success was hugely truncated when 'HAES741' was

grafted onto the seedlings and cuttings of Wild *M. jansenii* and *M ternifolia*. Though IGS of "M.tet1" seedlings was high (~60%), but was reduced significantly during final count (~25% FGS).

Among the existing cultivars, seedlings and cuttings of A268 had greater graft success. Seedlings of D4 and Daddow and Cuttings of A4 also had higher rates of graft success. Among the cultivars, A268 seedlings and cuttings produced more grafted trees than H2; and cuttings of D4 and Daddow also had greater graft success rates than H2 for scion cultivar HAES741. It was observed that graft success was higher in genotypes with greater stem diameter increase rate (GRD), which was also supported by the finding of Mng'omba et al. (2010) in mango. The lower rate of graft success in cuttings may be due to their inferior root system than that of seedlings ((Bell and Bell, 1993).

Establishment success in the rootstock trial

Establishment success of the rootstock trial is presented in Table 3. Initial establishment success was evaluated six weeks after planting. Most of the genotypes had 100% established success in the field trial with an average of 99% in seedlings and 98% in cuttings. Only 1 grafted seedlings of 'HAES842' and an 'A268' grafted cutting were died.

Table 3 Summary of grafted tree plantation and tree establishment in macadamia rootstocks used in Small tree high productivity trial at Nambour (on 15/05/2018)

Rootstocks	**Types**	**Seedlings**			**Cuttings**		
		NGT	**GTP**	**PTE**	**NGT**	**GTP**	**PTE**
842	High Performing Rootstock Cultivars	9	3	67	10	2	100
A16		9	4	100	2	-	
A268		7	5	100	10	3	67
A4		14	10	100	4	1	100
Beaumont		15	13	100	15	7	100
H2		21	12	100	5	2	100
BHI1	High Index Value	10	6	100	10	2	100
BHI2		8	1	100	9	7	100
BHI3		6	3	100		-	
D4	High yield efficiency		-	-	8	5	100
BHY1		10	10	100	10	2	100
BHY2		10	3	100		-	
BHY3			-	-	7	2	100
BHY4		2	1	100	4	-	
BHY5			-	-	6	1	100
BDW1	Potential Dwarves	10	5	100	2	-	
BDW2			-	-	8	-	
BDW3		2	-	-	9	1	100
BDW4		14	4	100	5	-	
IMCDW		10	3	100	3	-	
Daddow	AVG Resistant	7	3	100	10	5	100
M.jan1	Wild Species:	20	3	100	13	1	100
M.jan2	*M. jansenii*	8	-	-	4	-	-
M.jan3		10	-	-	7	-	-

M.ter1	Wild Species:	1	-	-	8	-	-
M.ter2	*M. ternifolia*	8	-	-	2	-	-
M.ter3		3	-	-	6	-	-
M.tet1	Wild Species:	16	4	100	6	-	-
M.tet2	*M. tetraphylla*	15	13	100		-	-
PaperShell	Others		-	-	5	1	100
Total		245	106	99	188	42	98

NGT= number of grafted tree, GTP= Grafted tree planted, PTE= Percent tree establishment.

Variability in growth parameters in grafted trees

Combined analysis of growth of grafted trees from November 2017 to May 2018 showed that there was significant genotypic variation in tree height increase. Significant variation between seedling and cuttings were observed for the growth of canopy width, canopy depth and shoot trunk circumference (Table 4).

Table 4. F-statistical probability estimated from REML mixed model

Components	**THI**	**CWI**	**CDI**	**RHI**	**RCI**	**SHI**	**SCI**
Genotype	0.039	0.168	0.858	0.372	0.248	0.929	0.189
Type (seedling vs cuttings)	0.146	0.01	0.004	0.102	0.913	0.112	0.009
Genotype x Type	0.935	0.367	0.353	0.824	0.798	0.921	0.574

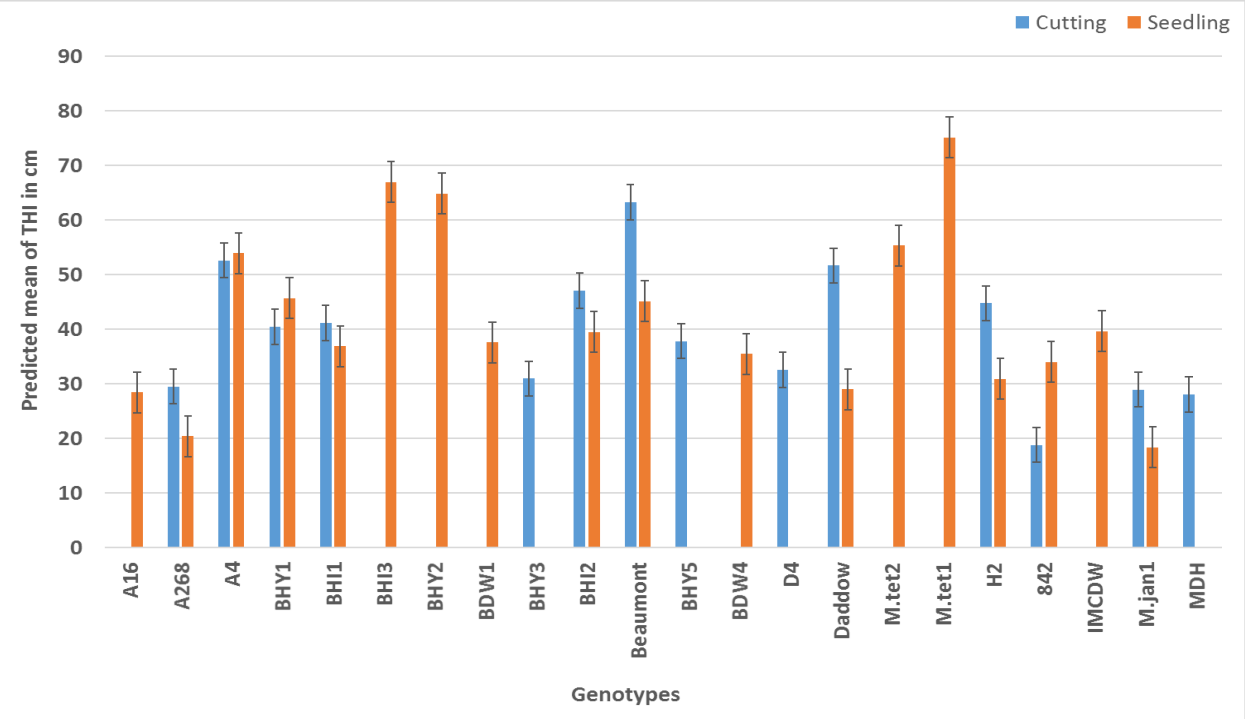


Figure 4. Variation in tree height increase (November 2017 to May 2018) in seedlings and cuttings of different genotypes

In total 21 genotypes were compared for growth parameters. Pairwise comparison between seedling and cuttings could be made only between 10 genotypes. THI varies from 19 cm to 63 cm in cutting and 18 to 75 cm in seedlings. Among seedlings, THI was greatest in "M.tet1" followed by "BHI3" and "BHY2". THI was least in the seedlings of "M.jan1" followed by 'A268'. In cuttings, grafted trees of "842" showed least vigour in THI and 'Beaumont" was greatest.

Variability in Flowering

In 2018, a total of 13 trees were flowered, of which five were grafted trees. Interestingly, all the grafted flowering trees have seedling rootstocks (BAMAM02-6-3, A268, A4, GTFRS00-23-30, and BALLO02-6-76) and non-grafted ones are cuttings of Beaumont and D4. In 2019, almost equal percentage of grafted and not grafted trees flowered (Table 5).

Table 5. Summary of flowering data of grafted and ungrafted trees.

Type	Number Flowering 2018	Number Flowering 2019	Total Plants
Grafted	5	80	142
Scion (HAES741)	0	0	4
Ungrafted	8	50	144
Total	13	130	290

REML mixed model analysis shows that there was no significant difference among the genotype and types (cuttings vs seedlings). But significant genotype X type interaction indicated that seedlings of some genotypes can be precocious than cuttings, and vice versa (Table 6).

Table 6. Wald statistics of fixed components.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Genotype	35.52	23	1.54	56	0.09
Type	3.99	1	3.99	56	0.05
Genotype x Type	22.42	9	2.49	56	0.02

Predicted flowering propensity of each genotype is presented in Table 6. Flowering propensity in cuttings varies from 0.09 (HAES842) to 1.42 (BAMAM02-7-23). While, in seedlings, BAMAM02-9-28 (1.77) shows the greatest potentiality of precocity and BBAFF 15-24 was the least. Interestingly, seedlings of genotype BALLO02-6-76 were precocious than cuttings, as suggested m genotype x type interaction (Table 7).

Table 7: Predicted means and standard errors (SE) of flowering propensity in the rootstock trial.

Genotype	Predicted mean		SE	
	Cutting	Seedling	Cutting	Seedling
MDH	0.77	*	0.51	*
BHY5	1.41	*	0.51	*

BHY3	1.42	*	0.37	*
D4	0.75	*	0.25	*
BHI2	0.80	1.77	0.22	0.53
Daddow	0.29	1.26	0.24	0.31
842	0.09	1.14	0.40	0.38
BHI1	0.11	1.12	0.37	0.22
BDW1	*	0.93	*	0.23
M.jan1	0.36	0.74	0.55	0.30
A268	0.91	0.74	0.37	0.27
BHI3	*	0.71	*	0.37
BHY2	*	0.71	*	0.38
BDW3	*	0.65	*	0.55
M.tet1	*	0.56	*	0.20
M.tet2	*	0.52	*	0.15
Beaumont	0.68	0.43	0.24	0.15
BDW4	*	0.42	*	0.27
A4	0.62	0.41	0.55	0.17
A16	*	0.38	*	0.27
BHY1	0.94	0.34	0.41	0.17
H2	0.97	0.30	0.38	0.16
IMCDW	*	0.30	*	0.30
BHY4	*	0.00	*	0.55

Conclusion

This study identified that the graft success depends on the genetic background of the rootstocks and compatibility with the scion. In comparison, seedlings were found to be more successful in grafting than cuttings, although one genotype outperformed other existing high performing cultivars for graft success as cutting rootstock. Establishment success of the rootstock trial was also high. Inclusion of wild germplasm increased the diversity in the rootstock trial. “M.jan1”, ‘A268’ and ‘A16’ seedlings are promising to reduce early vigour in grafted trees. Cuttings of “842” had also reduced total height of the grafted trees. We also presented the variability in early flowering among the rootstock genotypes. To select superior rootstock for high performance, we still need to continue the evaluation of growth and productivity parameters for the next five years. So, the following tasks are yet to be done:

- Management of rootstock trial sites over the next five years.
- Characterising for growth, flowering and yield parameters.
- Superior rootstock selection for high performance.
- Identifying the mechanism of rootstock-scion interaction.

Appendix 2.2: Report on anatomical structure associated with vegetative growth variation in macadamia

Benjamin D. Toft, Mobashwer M. Alam and Bruce L. Topp

Introduction

For macadamia, vegetative vigour and canopy size are limiting factors of both tree productivity (yield efficiency) and planting density (yield per hectare). The development of low-vigour macadamia genotypes would provide breeding material for beneficial new scion and rootstock cultivars. Evaluation of tree vigour and productivity takes many years and therefore the breeding process is slow for such traits.

Traits that are correlated with vigour will be useful in selection of seedlings. Across species, xylem diameter variations relate linearly to sap flow rate (Sevanto et al., 2008). In peach, vigour-reducing rootstocks have been associated with decreased hydraulic conductance due to reduced xylem vessel diameter compared to vigour-inducing rootstocks (Tombesi et al., 2010). It has been suggested that xylem vessel diameter can be useful in the early selection of dwarfing rootstock genotypes (Bruckner and DeJong, 2013).

Although trials are currently underway in macadamia to find dwarfing rootstocks and vigour-reduced genotypes, little is known of the relationship between vascular traits and vigour. There is high variability in tree size among genotypes in existing macadamia trials. This study aims to find relationships between tree size and vascular traits, which may be used for early selection of dwarf genotypes.

Materials and methods

Sampling and vigour measurements

Five cutting-grown replicates of three genotypes were selected from a breeding trial planted in November 2011 at Maroochy Research Facility, QLD, Australia (26°38'39.8"S, 152°56'14.2"E, elevation 59m). 'D4, 'B25' and 'B63' were selected as exhibiting high, medium and low vigour, respectively. The trial was located within an orchard of 859 trees planted at 4 m between rows and 1 m between trees within rows (~2500 trees ha^{-1}). Each clonal replicate was surrounded by neighbouring trees of different genotypes.

Tree size and vascular measurements

Tree height was measured each year from 2013 to 2018 (excluding 2017), and the change in tree height from 2013 to 2018 was considered an indicator of vigour.

Vascular traits were measured in autumn 2018 on three subsampled branches per tree. Shoot samples were taken from the most recent mature apical flush on the primary axis of each subsampled branch. To be considered mature, the apical bud was no longer extending and the shoot was no longer green. Shoot samples were taken from the second most apical internode at the midpoint between the nodes. Sections of ~5 mm were fixed in acetone for 2 days before sectioning.

Sectioning of the shoot samples were prepared using a Thermo Scientific CryoStar™ NX70 Cryostat. Sections were cut 80µm thick, using specimen and knife temperatures of -17ºC and -22ºC, respectively.

A MetaSystems Metafer VSlide Scanner was used to image whole cross-sections at 5x by combining multiple fields of view in Zeiss Axio Imager Z2. Multiple fluorescence filters were used (DAPI, GFP, Cy5) to provide differentiation in tissue using the natural luminescence of the plant cells under fluorescent light. Some samples provided unclear

imaging due to difficulty in the preparation of whole cross-sections and therefore were not included in the analysis.

Images were analysed using Fiji (Schindelin et al., 2012). Using the tools within Fiji the shoot cross-sectional area (CSA), number of vascular bundles and total area of the pith was measured manually for each shoot section. A simple macro was written in Fiji to automatically count and measure the area of all xylem vessels over 150 μm^2 with a circularity of 0.5 and above (Fig. 1). This data was used to calculate total vessel number, mean vessel size, total additive vessel area per cross-section (μm^2) and pith area (μm^2). For vessel size, this analysis only considered the mean vessel area of the ten largest vessels per shoot section.

Statistical analysis

Statistical analysis was performed using R (R Core Team, 2018). Unbalanced ANOVA was performed to determine significant differences between genotypes for all traits, as some samples were unable to be analysed. Linear regression was calculated to demonstrate relationships between vascular traits and change in tree height over time.

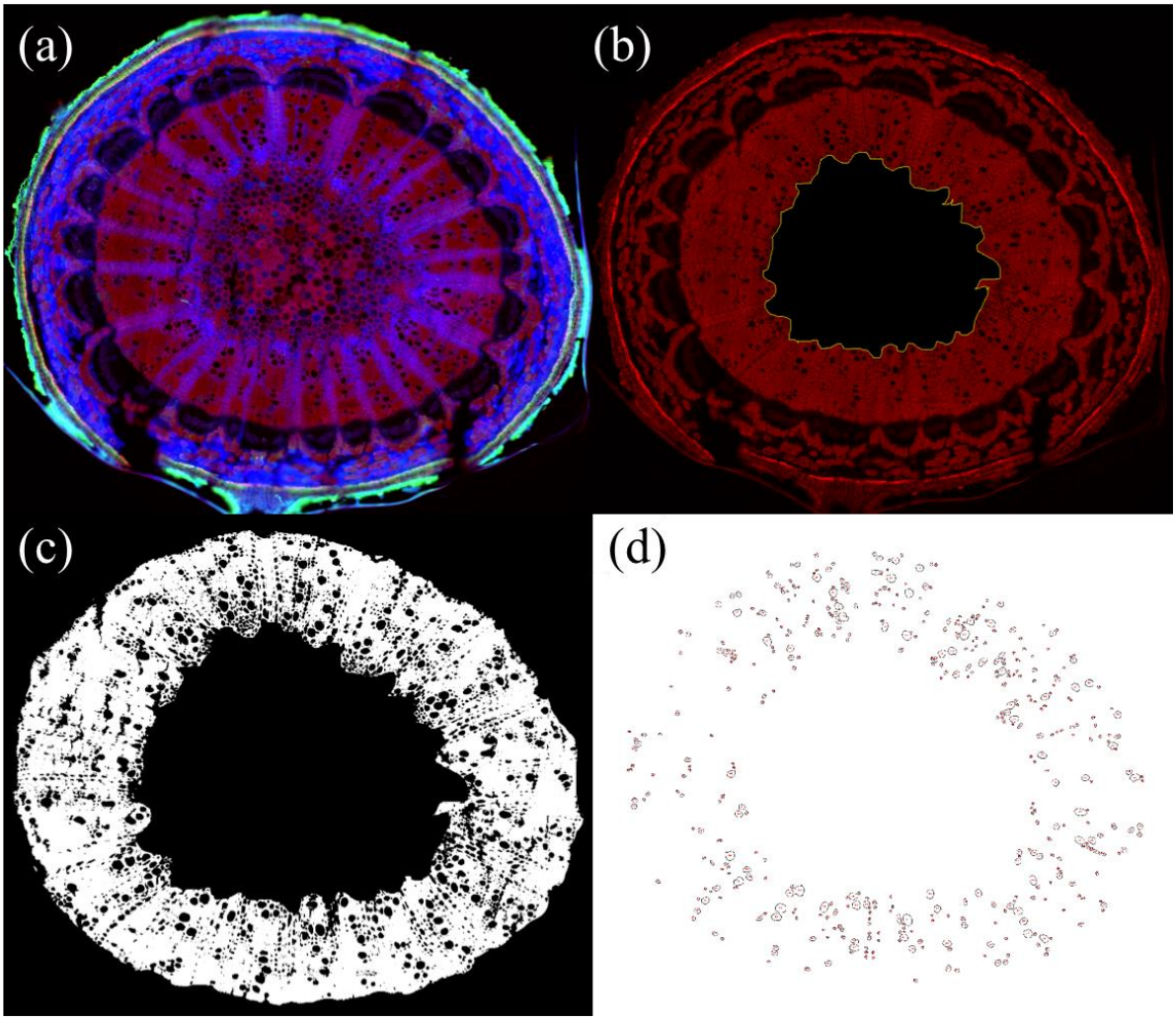


Figure 1: Process of measuring vascular traits in Fiji. (a) defining tissues by overlaying images using three fluorescent filters; (b) measuring and removing pith area; (c) measuring outer area of xylem tissue and removing phloem and outer tissues, and increasing colour threshold to define xylem vessels; (d) applying macro that counts and measures

the area of each xylem within parameters of minimum size and circularity.

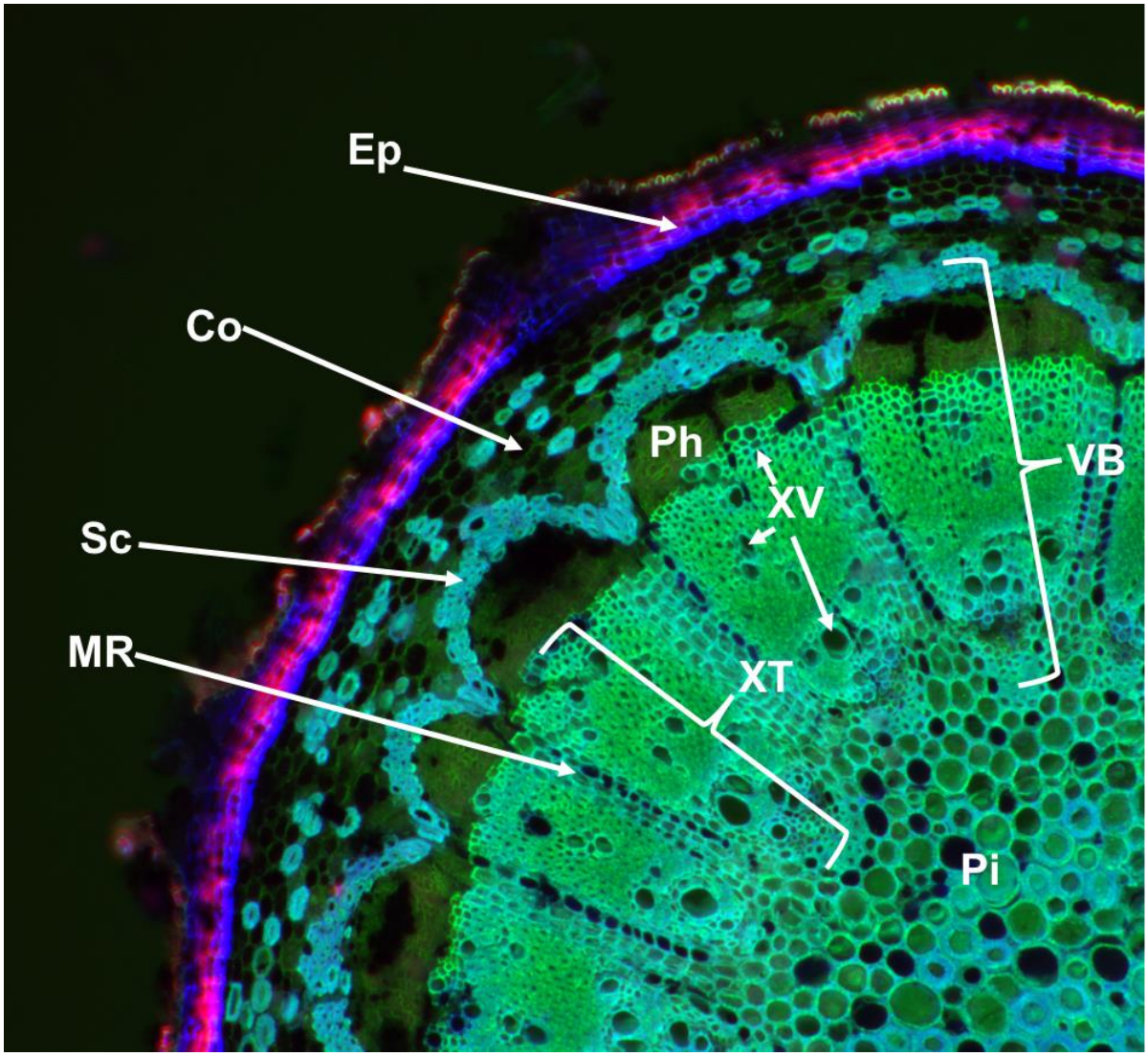


Figure 2: Cross-section of a macadamia shoot under fluorescence microscope with no staining. Ep, epidermis; Co, cortex; Sc, sclerenchyma; MR, medullary rays; Ph, Phloem; XT, xylem tissue; XV, xylem vessels; VB, vascular bundle; Pi, pith.

Results and discussion

Genotypic differences in vigour

Vigour over time was measured as change in tree height between 2013 and 2018; there were significant differences in vigour over time between the three genotypes ($P < 0.001$). The mean tree height in 2018 was 5.98 m for 'D4', 4.13 m for 'B25' and 2.09 m for 'B63' ($P < 0.001$). For genotype 'B63' growth rate was slow over time (Fig. 3), contrasting with 'D4' which had a higher rate of growth over time. 'B25' grew quickly from 2013-2016 but at a slower rate from 2017-2018 resulting in an intermediate tree height at 2018. Trunk cross-sectional area related to change in tree

height (R^2 = 0.97, P < 0.001).

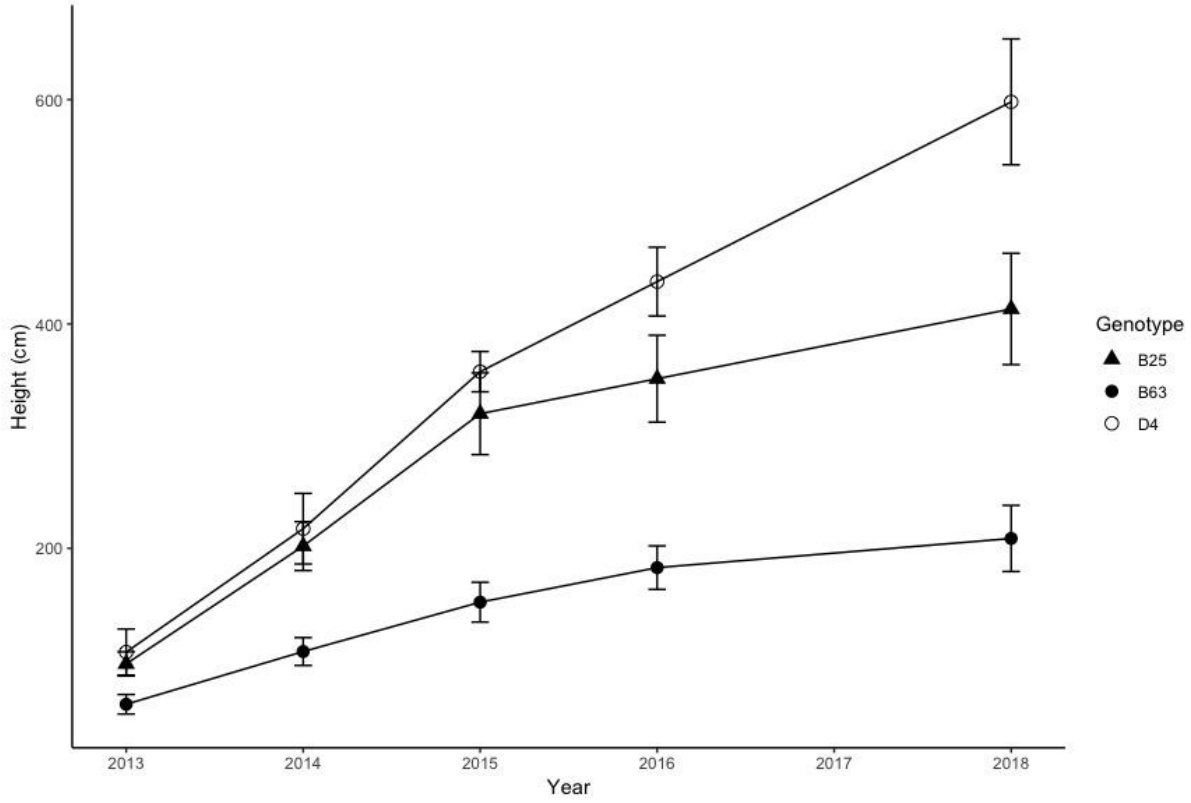


Figure 3: Mean change in tree height (cm) of three genotypes 'D4' (high vigour; empty circles), 'B25' (medium vigour; full triangles) and 'B63' (low vigour; full circles). Trees planted in 2011, data shows yearly height from 2013 to 2018, no data for 2017.

Genotypic differences in vascular traits

The mean CSA of shoots at the midpoint of the second internode of the most recent flush was 7.4, 11.3 and 14.4 mm^2 for 'B63', 'B25' and 'D4', respectively. Shoot cross-sectional area (CSA) was significantly different between 'D4' and 'B63' (P < 0.001), and between 'B25' and 'B63' (P < 0.05), but not between 'D4' and 'B25' (P = 0.09). It is interesting to note that for shoots with three or four leaves at a node, pith shapes tended to be triangular or square, respectively. Examples of cross-sections for each genotype are shown in Fig. 4. The mean area of the ten largest xylem vessels in each section was 921, 1190 and 1368 μm^2 for 'B63', 'B25' and 'D4', respectively (P < 0.001). The total additive vessel area for each cross-section was 0.056, 0.132 and 0.178 mm^2 for 'B63', 'B25' and 'D4', respectively (P < 0.001). Pith area was double that in 'D4' than in 'B25' (P < 0.01).

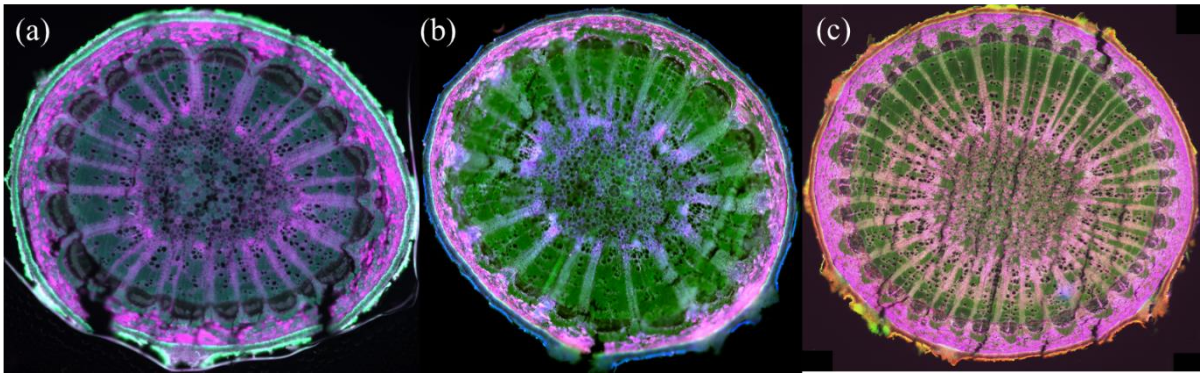


Figure 4: Examples of shoot sections at the midpoint of the second internode of the most recent flush for (a) 'B63' (three leaves); (b) 'B25' (three leaves) and (c) 'D4' (four leaves).

Relationships between vigour and vascular traits

Tree-scale vigour (change in tree height over 5 years) related to the mean xylem vessel size (ten largest vessels) in a positive linear fashion (R^2 = 0.57, P < 0.001). Within this relationship, there was a clear separation of the tree genotypes for both vigour and vessel size (Fig. 5). There were also positive linear relationships between vigour and pith size (R^2 = 0.70, P < 0.001), and vigour and total vessel number (R^2 = 0.40, P < 0.01). When considering the percentage of CSA that consisted of xylem vessel space (total additive vessel area / CSA) there was also a positive relationship with vigour at the tree scale (R^2 = 0.41, P < 0.01).

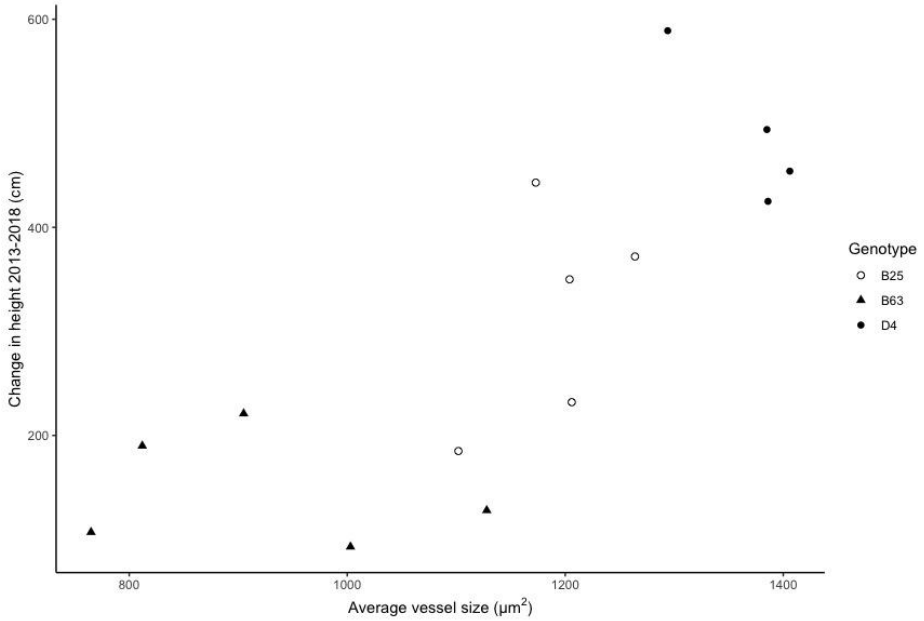


Figure 5: Positive linear relationship between mean area of the ten largest xylem vessels in the most recent flush and the change in tree height over 5 years. $R^2 = 0.61$, $P < 0.001$. Genotypes 'B63' (full triangle), 'B25' (empty circle) and 'D4' (full circle) group based on tree size and vessel size.

Conclusions

This study suggests that variability in xylem vessel size in the most recent flush is related to variability in vigour at the tree scale. From this study we can hypothesise that decreased hydraulic conductivity in smaller vessels may limit vigour in low-vigour cultivars such as 'B63'. Further research is required to determine differences in hydraulic conductivity in the sample population. Further studies that determine the ability to estimate long-term vigour from young shoot xylem vessel size in macadamia may allow faster selection of dwarf cultivars in breeding programs.

Appendix 3: Economic evaluation of the macadamia breeding program: Comparison of new selections with standard industry varieties

Disclaimer

Results presented in this report are based on yield and kernel recovery data from macadamia regional variety trials.

The project partners associated with this report include the Queensland Alliance for Agriculture and Food Innovation and the Department of Agriculture and Fisheries. While every care has been taken to ensure the validity of information collected and analyses produced, Neither of these project partners, nor any persons acting on their behalf, make any promise, representation, warranty or undertaking in relation to the appropriateness of findings in this report and expressly disclaim all warranties (to the extent permitted by law) about the accuracy, completeness, or currency of information in this report.

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Introduction

The following sections compare the relative economic performance of recent releases from the macadamia breeding program and regional variety trials. These 20-year cash flow forecasts compare both annual net cash flow and cumulative cash flow for the four recent variety releases (G, J, P and R) and compares these with established industry varieties A16 and 741. Forecasts include performance in both the Bundaberg region and the Northern Rivers of NSW.

All scenarios model establishment of a new 100-hectare farm. Irrigation establishment and operating costs are included in all Bundaberg scenarios and excluded from all Northern Rivers scenarios.

The industry average planting density 312 trees per hectare (8x4m) is assumed for all varieties other than P, which is based on the higher density of 400 trees per hectare due to the relatively small size of these trees.

This analysis has been undertaken to evaluate elite selections from the B1.1 populations in regional variety trials.

Varieties and assumptions

The following tables show yield, kernel recovery and planting densities for new varieties and industry standards from regional variety trials. Growth curves were developed for each variety using measured yield from years 1 to 8. Mature yield was estimated for each variety by extrapolating these growth models to full maturity.

Table 1: Performance of varieties from regional variety trials in Bundaberg

Variety	G	J	P	R	A16	741
Estimated mature NIS yield (T/ha)	7.52	7.07	9.99	6.1	6.56	6.78
Saleable kernel recovery %	38.20	41.10	32.60	35.20	37.20	34.90
Trees / ha	312	312	400	312	312	312

Table 2: Performance of varieties from regional variety trials in Northern Rivers NSW

Variety	G	J	P	R	A16	741
Estimated mature NIS yield (T/Ha)	8.33	6.01	9.78	8.2	6.60	6.40
Saleable kernel recovery %	39.5	41.3	32.8	36.1	38.6	37.4
Trees / ha	312	312	400	312	312	312

Table 3: Assumptions for financial analysis scenarios. Price growth, inflation, depreciation, finance and taxation have been excluded to simplify analysis.*Average cost data from benchmark cost of production sample 2013-2017.

Assumptions	Bundaberg (irrigated)	Northern Rivers NSW (non-irrigated)
NIS price (10-year fixed average @33% KR)	$3.80	$3.80
Farm establishment costs	$4,180,000	$4,002,500
Non-bearing fixed costs per hectare*	$2,715	$1,657
Bearing costs per hectare*	$7,798	$6,693

Rates of return

The following tables show the net present value (NPV) and internal rate of return (IRR) for selections in both Bundaberg and Northern Rivers NSW.

NPV is calculated by subtracting the present value of the capital outlays from the present value of the cash inflows. IRR is the rate of return, or discount rate at which the net present value (NPV) will be equal to zero.

New varieties P, J and G achieved a higher NPV and IRR than standard varieties 741 and A16 in Bundaberg. New variety R achieved the lowest NPV and IRR of the six varieties analysed for Bundaberg. NPV and IRR was highest for three new varieties (P, J and D) in Northern Rivers NSW, however variety J showed a lower NPV and IRR than standard variety A16.

All varieties (including standards) achieved a higher NPV and IRR in Northern Rivers NSW compared with Bundaberg. This may be explained by the lower farm establishment and production costs for non-irrigated farms in Northern Rivers NSW.

Table 4: NPV and IRR for new and standard varieties in Bundaberg

Variety	NPV	IRR
P (HD)	$11,786,344	13.3%
J	$10,269,956	12.7%
G	$10,066,610	12.7%
741	$7,355,470	11.0%
A16	$6,578,898	10.3%
R	$5,850,380	9.9%

Table 5:NPV and IRR for new and standard varieties in Northern Rivers NSW

Variety	NPV	IRR
P (HD)	$13,933,636	15.3%
G	$13,766,906	15.3%
R	$12,296,133	14.8%
A16	$9,561,524	12.9%
J	$9,130,651	12.6%
741	$8,659,837	12.3%

Cash flows

The following figures show both annual net cash flow (NCF) and yearly cash balance (YCB) for varieties G, J, P and R compared with established industry varieties A16 and 741 in Bundaberg and Northern Rivers NSW.

NCF is the difference between annual cash inflows and outflows over the analysis period. In the following scenarios, this is the difference between annual income generated from nut production and costs incurred from various heads of expenditure. Yearly cash balance or YCB is the cumulative net cash flow over the analysis period.

In Bundaberg varieties P and J achieved positive cash flow sooner than standard varieties 741 and A16. Over the term of the analysis varieties P, J and G maintained higher NCF than the two standards. These results are reflected in the YCB for varieties in Bundaberg (Figure 3).

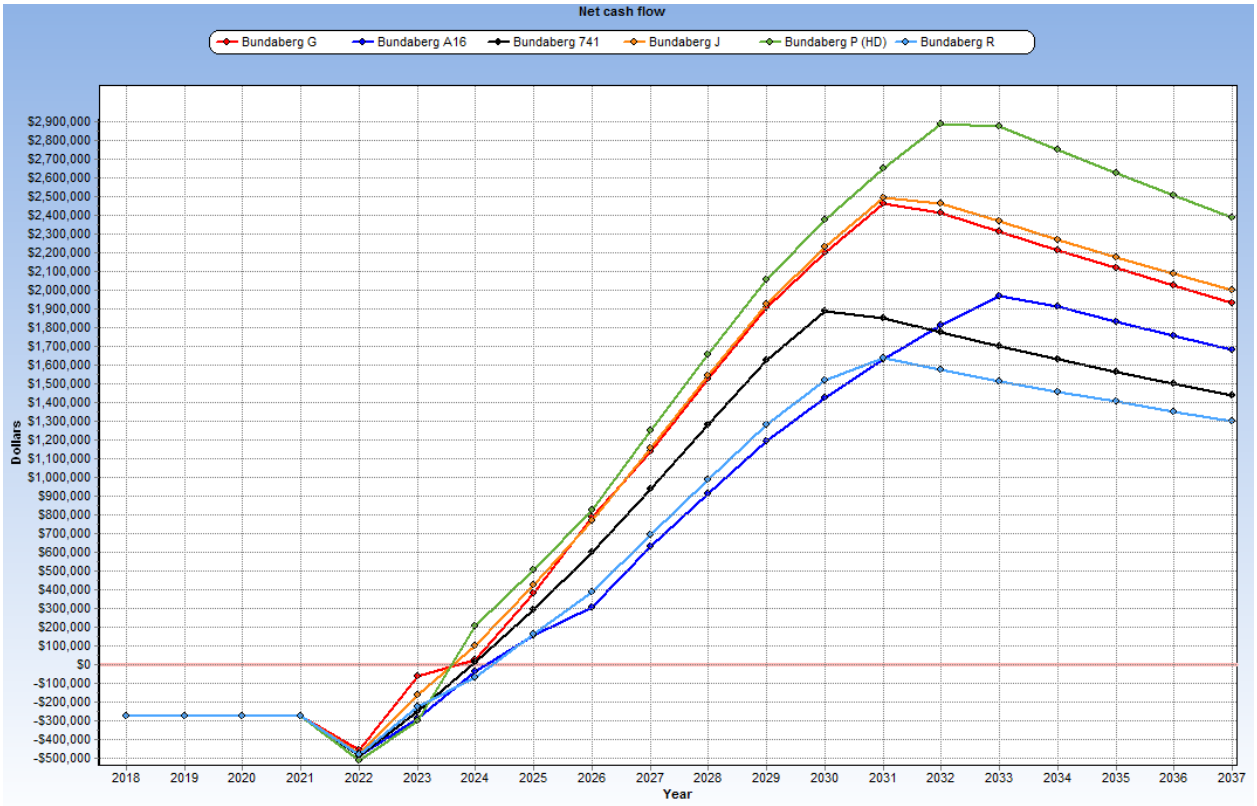

Figure 1: Net cash flow by variety for Bundaberg

In Northern Rivers NSW positive cash flows were realised earlier for varieties P, G and R when compared to standard varieties. NCF for variety J over the analysis term was very similar to standard varieties A16 and 741, while P, G and R remained higher. These cash flows are shown cumulatively in the yearly cash balance for varieties in Northern Rivers NSW (Figure 4).

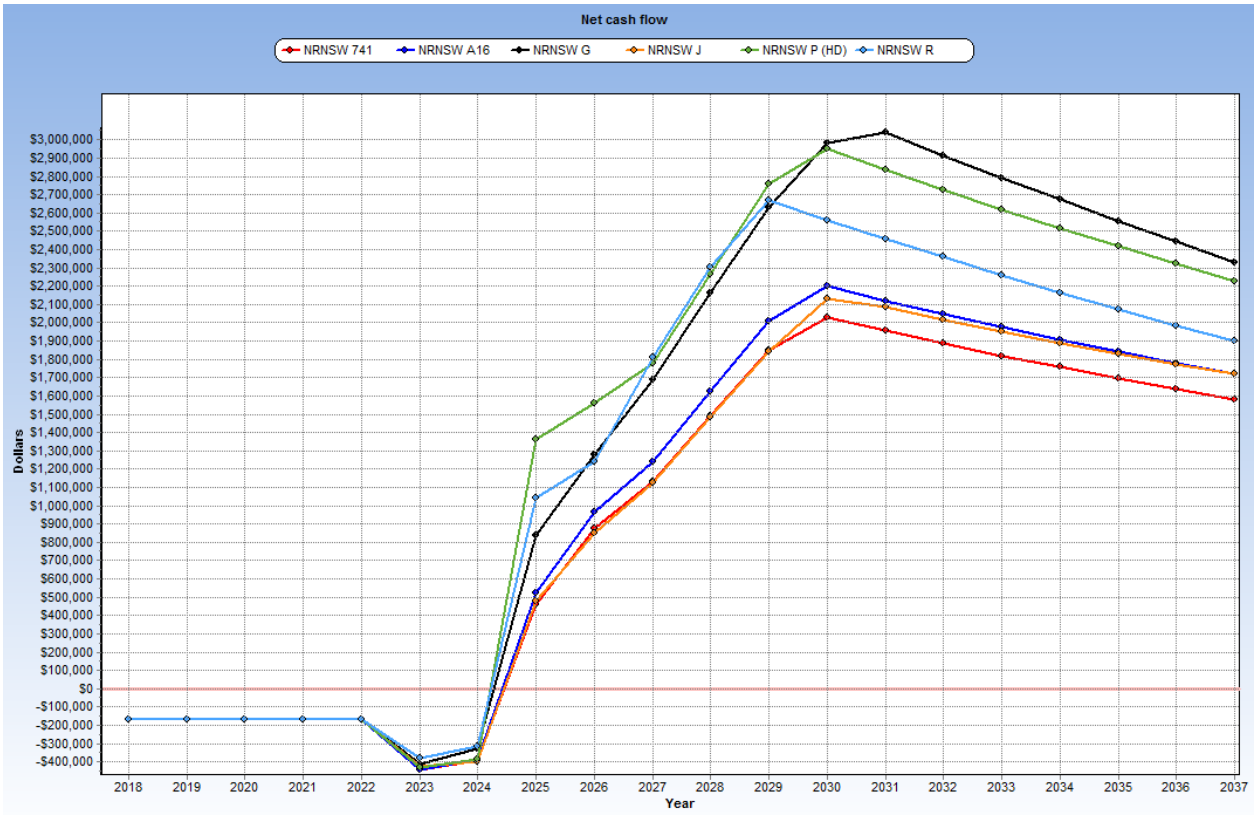

Figure 2: Net cash flow by variety for Northern Rivers NSW

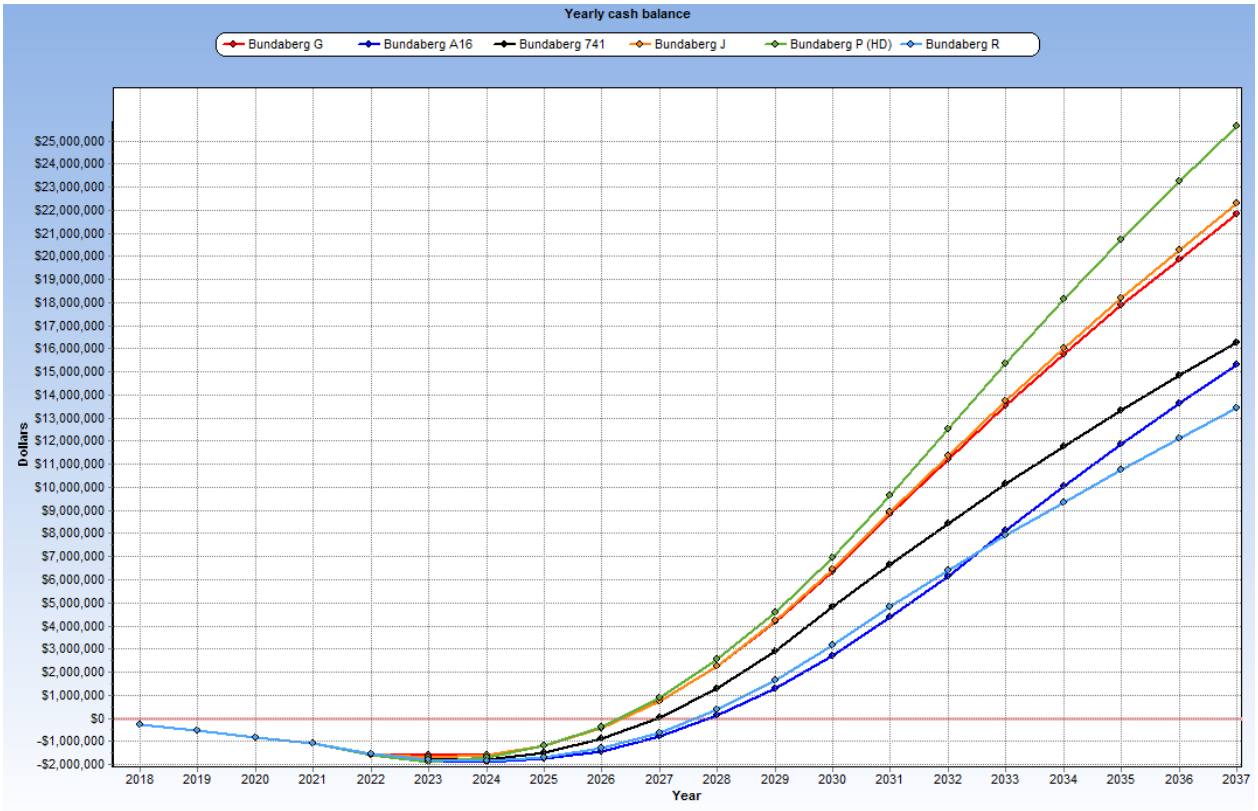

Figure 3: Cumulative cash balance by variety for Bundaberg

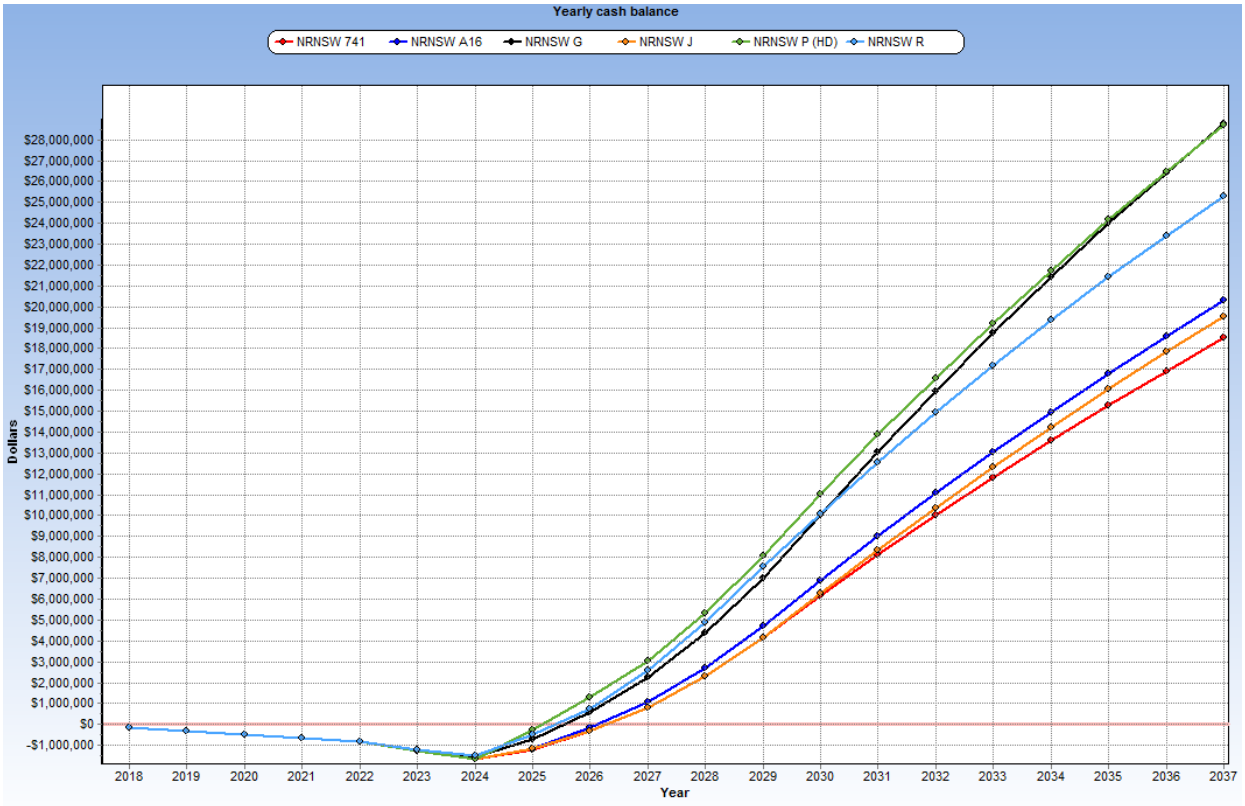

Figure 4: Cumulative cash balance by variety for Northern Rivers NSW

Summary

Based on available yield and kernel recovery data to date, analysis of the four newly released varieties has identified their long-term economic potential compared with industry standard varieties.

While it is apparent that varieties perform differently in different production regions, these analyses show that increasing yield and kernel recovery can result in significant long-term economic gains and farm business viability.

Appendix 4: Screening of macadamia cultivars, progeny and wild germplasm for husk spot resistance

Mobashwer Alam, Jasmine Nunn and Bruce Topp

Introduction

Husk spot disease, caused by the fungal pathogen *Pseudocercospora macadamiae,* can induce significant premature fruit abscission in Macadamia. Management of the disease adds to farm costs, though in the absence of proficient control, saleable kernel and subsequently, farm profits can be significantly reduced due to increased proportions of immature nuts. Historically, resistance to pests and diseases has not been a major focus in macadamia breeding. Therefore, in the current macadamia breeding project (MC14000), we aimed to screen a large number of breeding progeny and germplasm. We are reporting the updates of the screening.

Materials and methods

Experimental materials and Site 1: Screening breeding population

The experiment is going on in the precocity trial (BNAMB11), which was planted at Maroochy Research Station, Nambour in 2011. The trial consisted of 986 tress including parents and progeny from a diverse group of cultivars from Hidden Valley Plantation (HVP) Beerwah Australia; Hawaiian Agricultural Experiment Station (HAES), Hawaii, USA; , Australian Heritage Cultivars (Aus Heritage), Elite selections from the Australian macadamia breeding program (Aus Elite) and *Macadamia jansenii* wild germplasm (Wild *jansenii*). The precocity trial was planted with the tree spacing of 1m×4m and incorporated the open-pollinated seedlings and maternal genotypes in an incomplete block design. The site was planted in an incomplete bock design consisting of 25 blocks where progeny of the families and 2-6 replications of parents were spatially distributed.

Experimental materials and site 2: Screening wild germplasm and cultivars

This experiment is going on in an ex-situ collection (GTAIRO00) of wild germplasm planted at Tairo, QLD in 2000. A total of 363 plants, including 212 wild accessions and 14 cultivars, are being evaluating for husk spot susceptibility.

Husk Spot inoculation

Macadamia pericarps with visible *P. macadamiae* lesions were collected from Bundaberg and Gympie orchards during July and August in 2017, 2018 and 2019. Approximately 75g of pericarps were placed into individual plastic netted bags, as previously published by Miles et al. (2010). The day before inoculation, pericarp bags were dipped in tap-water for 10 seconds before being placed in an enclosed tub at room temperature for 24 hours in order to maintain humidity and activate conidia on lesions. Bags were then hung above nutlets in datum trees. Positions were chosen to capture the maximum number of match-head to pea-size nutlets possible within the rain splash zone under each bag. At BNAMB11, overhead sprinkler irrigation was constructed in order to mimic rainfall over most of the inoculated trees. Irrigated blocks received overhead irrigation for 30 minutes at 6pm 3 times weekly for 4 weeks, until all nutlets were pea-size or larger. Irrigation was set at this time of day in order to avoid conditions favourable for evaporation, so nutlets could stay wet long enough for germination of conidia to occur. Considering the expense of the setting up overhead sprinkler, we are evaluating the GTAIRO00 trial under natural rainfall condition.

Screening design

BNAMB11 trial was first carried out over the 2017-2018 nut bearing season on a subset of trees, then repeated with

an increased number of trees over the 2018-2019 season. Blocking of the trial was determined according to variation in field soil within the trial. Overhead irrigation was provided in both years for the month of November. For the second year, husk bags from the previous year were left in trees, thus trees included in both years had 7 bags in the second year, whereas trees that were added in year two only received 3 bags. Additionally, in year two, net bags were gently placed over inoculated fruit and pegged to branches. Abscised fruit were collected from within these bags. In order to avoid having the netted bags catch and pull on the fruit, the bags were placed over fruit in mid-December once they had reached nut-in-shell size or larger so the pointed micropile end of the husk had smoothed out. In the second season, some of the newly added clonal trees were located outside of the overhead irrigation. Differences in numbers of husk bags and in irrigation were captured in blocking. GTAIRO00 trail is being conducting in 2019-2020 nut bearing season. Further details of both years are recorded in Table 1.

Table 1: Details of inoculation and sampling of husk spot screening trial over nut-bearing seasons 2017-2018 and 2018-2019.

	2017-2018	**2018-2019**	**2019-2020**
Number of trees inoculated	85 progeny, 28 families, 17 parent genotypes, 106 trees in total	361 progeny, 31 families, 24 parent genotypes, 454 trees in total	212 wild accessions and 14 cultivars, 360 trees in total
Overhead irrigation	All trees	Most trees. Some clonal cultivars did not receive overhead irrigation.	None
Number of husk bags inserted per tree	4	3 or 7	5
Date of insertion of new husk bags	6th October 2017	24th October 2018	October 2019
Method of collection of abscised nuts	From ground.	From within netted bags.	

Phenotyping

For the 2017-2018 season, phenotyping was carried out on the 30th of January and the 23rd of March in 2018. For the 2018-2019 season, phenotyping was performed on the 19th of February, the 18th of April and on the 20th of May in 2019. For the 2018-2019 season, phenotyping will be performed on the February- May in 2020.

For each datum tree, the following data was recorded on fruit located within the splash zone of inoculation bags:

- Number of non-abscised nuts with one or more lesion,
- Total number of non-abscised nuts,
- Number of abscised nuts with one or more lesion, and
- Total number of abscised nuts.

Preliminary analysis

Preliminary analyses were undertaken. Means of percentage infected nuts were calculated for each family and parent using Genstat v.18. For parents, REML analysis was undertaken with total nuts assessed as a covariate. For

families, ANOVA was undertaken with blocking as a factor and total nuts assessed as a covariate. Year, harvest date and other treatments were not considered at this time.

Final analysis

The method for final analysis is currently being researched. Several issues must be considered in order to provide robust results. These are summarised as follows:

- Binomial (infected or not infected) data converted to proportions.
- Large variation in sample size (number of nuts assessed) between trees and dates.
- Repeated measures. Data was collected at more than one time.
- For later months, data was censored due to abscission of fruit between dates.
- Trees received different numbers of husk bags and some were irrigated and some were not.

Preliminary results

The minimum and maximum percentages of infected nuts in parents were 75.18% and 96.08% respectively. Differences between families were not significant (F=0.64; P=0.897) (Table 1). The minimum and maximum percentages of infected nuts in families were 69.22% and 101.42% respectively. Significant differences between some families were found (F=2.3; p<0.001) (Table 2).

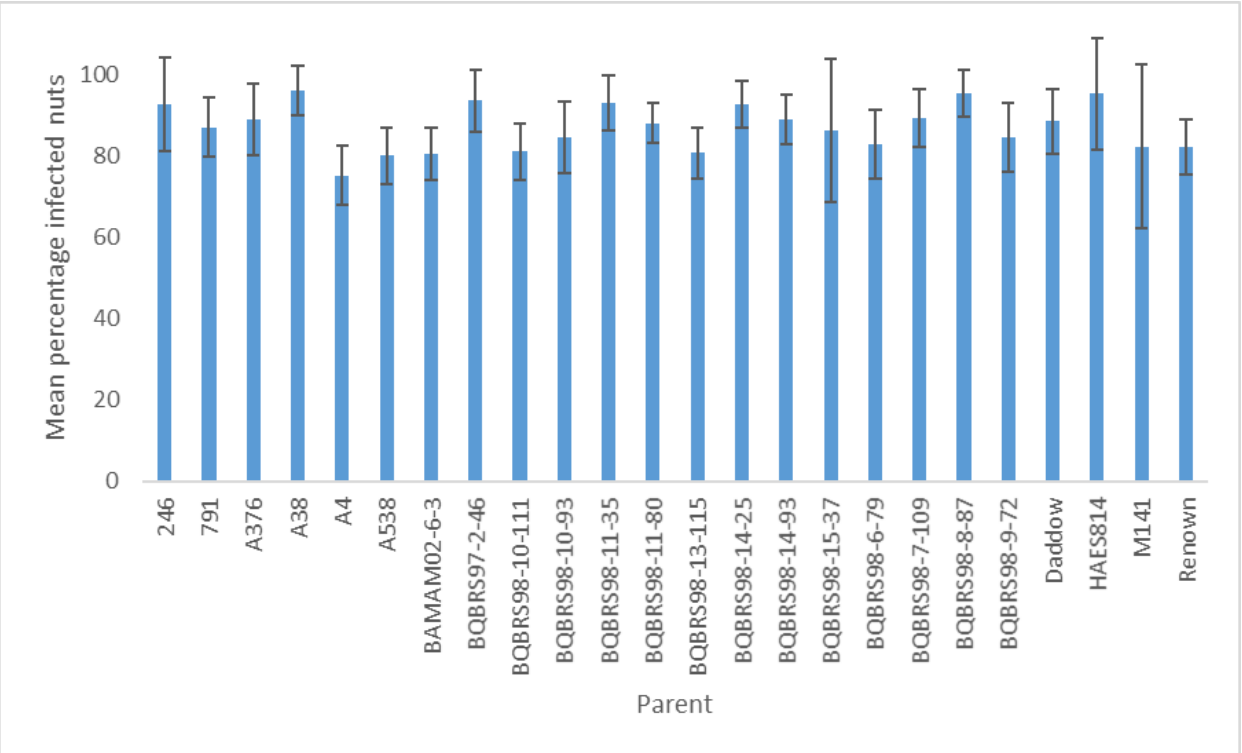

Figure 1: Percentage infected nuts of parents. Bars indicate standard error.

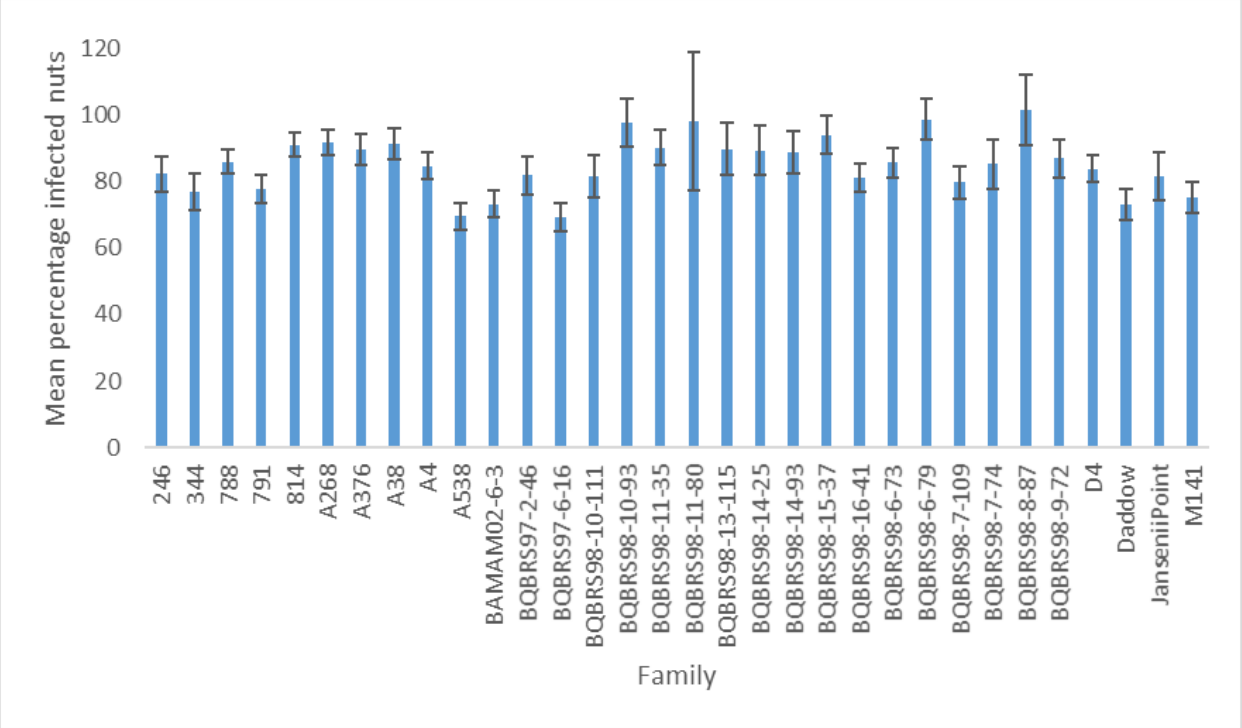

Figure 2: Percentage infected nuts of families. Bars indicate standard error.

Conclusion

This study was aimed to find out potential sources of husk spot resistance in macadamia. To this aim, we screened a large number of cultivars, progeny and wild germplasm. Considering the importance of this project, we converted it into a PhD project. Ms Jasmine Nunn started her PhD in January 2019. Screening process is being continued and will be completed in December 2021.

Table S1: List of cultivars and progeny screened in 2017-2018 and 2018-2019 nut bearing seasons for husk spot resistance in Macadamia

Cultivar	2017-2018		2018-2019	
	Progeny	Clone	Progeny	Clone
246	3	-	13	2
344	1	-	13	-
788	4	-	15	-
791	4	1	14	3
814	6	-	13	1
A268	6	-	11	-
A376	4	1	15	2
A38	4	-	12	6
A4	5	1	14	4
A538	4	1	13	4
BAMAM02-6-3	3	1	13	5
BQBRS97-2-46	1	1	11	3
BQBRS97-6-16	3	-	13	-
BQBRS98-10-111	4	2	13	4
BQBRS98-10-93	1	1	7	3
BQBRS98-11-35	3	2	13	5
BQBRS98-11-80	0	2	1	7
BQBRS98-13-115	1	1	7	5
BQBRS98-14-25	0	1	11	6
BQBRS98-14-93	2	1	9	6
BQBRS98-15-37	1	-	13	2
BQBRS98-16-41	4	-	17	-
BQBRS98-6-73	3	-	14	-
BQBRS98-6-79	1	1	6	4
BQBRS98-7-109	2	1	11	4
BQBRS98-7-74	-	-	9	-
BQBRS98-8-87	0	2	8	5
BQBRS98-9-72	2	-	12	3
D4	3	-	13	5
Daddow	5	1	16	3
Macadamia jansenii (point)	1	-	-	-
M141	3		11	1
Total	84	21	361	93

Table S2: List of cultivars and wild germplasm is being screened in 2019-2020 nut bearing seasons for husk spot resistance in Macadamia

Cultivar/wild germplasm	**Plants**
2/12 Mc	2
2/48 B	1
246 X OP	6
4/7 Mc	3
A199	5
A38	3
A4	2
A9/9	1
C3	2
Fuji; HAES 791	1
Ikaika; HAES 333	3
NG29	2
NG35	3
NG8	1
Planted germplasm tree	38
Unknown tree	2
Wild *M. integrifolia*	149
Wild *M. jansenii*	5
Wild *M. ternifolia*	2
Wild *M. tetraphylla*	74
Wild tree from mixed/hybrid population	42
Wild tree of uncertain origin	16
Grand Total	**363**

Appendix 5: Summary of work from Thuy Mai's PhD study: Genomic-assisted exploitation of wild germplasm for improvement of macadamia

Appendix 5.2: Phenotypic characterisation of wild macadamia germplasm

This chapter is still in progress and aims to:

- describe and analyse the morphological variation of wild germplasm in Tiaro vegetative, floral and nut traits
- determine the correlation between the phenotypic traits
- evaluate their potential in macadamia breeding

The study site is the ex-situ germplasm plantation at Tiaro (QLD). This site was established in 2001 with cuttings collected from wild and planted macadamia populations. The study involved 540 trees of 329 accessions.

Various phenotypic traits were collected from each tree from 2017 to 2019 (see Table 1). An average of 50 nuts per tree were collected to measure the nut traits, including individual nut-in-shell weight, individual nut-in-shell length and width, shell thickness, kernel weight and kernel recovery. Of the 540 trees, 372 produced nuts in 2017. The experiment was repeated in 2018.

Trees were ranked from highest to lowest yield in 2017 per species. An average of ten high-yielding accessions per species (except for *M. jansenii* with four and *M. ternifolia* five accessions) were chosen to study nut drop pattern. These trees were harvested once per month from March to September in 2018 and 2019 to determine the nut drop pattern. Dried nut-in-shell weight was used to compare the nut drop pattern between the species and among the accessions within species.

Data analysis is currently being performed in ASReml-R to estimate the genetic effects for single traits, estimate heritability, and correlation among traits.

Table 1: List of phenotypic traits collected from germplasm collection, and years that data were collected

Traits	**2017**	**2018**	**2019**
Growth traits			
Tree height	v	v	
Trunk circumference	v	v	
Canopy length	v	v	
Canopy width	v	v	
Skirt height	v	v	
Leaf traits			
Number of leaves per whorl	v		

New flush colour	v		
Leaf margin serration	v		
Nut traits			
Nut weight	v	v	
Nut length	v	v	
Nut width	v	v	
Shell thickness	v	v	
Kernel weight	v	v	
Kernel recovery	v	v	
Flower traits			
Flower colour	v		
Flowering time (0 – 50 – 100% blooming)	v	v	
Stick-tight	v		
Nut drop pattern		v	v

Appendix 5.3: Identification of genetic markers linked to economically important traits

This chapter aims to identify genetic variants developed in chapter 3 associated with the phenotypic data collected in chapter 4 by a genome-wide association study (GWAS) using a mixed linear model (MLM) implemented with the bioinformatics software to test the hypothesis if there are some genetic markers related to the interested traits in macadamia.

308 wild accessions were genotyped by the set of 2,872 SNP markers and 8,415 silicoDArT markers available from Chapter 1: Population structure and genetic diversity. This data will be combined with phenotypic data collected in Chapter 2. Genome-wide association studies (GWAS) for the main traits such as tree height, trunk circumference, nut size, shell thickness and kernel recovery will be performed using ASReml-R.

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