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High Outcrossing Levels among Global Macadamia Cultivars: Implications for Nut Quality, Orchard Designs and Pollinator Management

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Abstract: Global fruit and nut yields are affected by shortfalls in pollinator populations, and pollen limitation is most prevalent among tropical, bee-pollinated and self-incompatible plants. Macadamia is a subtropical, bee-pollinated crop in which some cultivars have been found to be highly outcrossing. We aimed to determine the extent of outcrossing and its effects on nut quality across a wide range of international macadamia cultivars in three countries. We sampled fruit from 19 macadamia cultivars across 23 sites in Australia, Brazil and South Africa. We used genotype-by-sequencing and MassARRAY methods to assign paternity to individual fruit and we assessed pollen-parent effects on nut quality. Macadamia was highly outcrossing, producing 80–100% of fruit by cross-pollination, at 17 of the 23 sites. Mixed mating (41–72% outcrossing) was identified at five sites, and low outcrossing (10%) was identified in one cultivar at one site where it was isolated from other flowering macadamia trees. Outcrossed fruit often had significantly better quality than selfed fruit, with 1.61–3.39 g higher nut-in-shell mass, 0.53–1.55 g higher kernel mass, 3.3–6.4% higher kernel recovery, and 3.0–3.5% higher oil concentration. The differences in kernel recovery equated to differences in value of USD 433–841 per ton of nut-in-shell at prices of USD 3000 per ton. In summary, macadamia cultivars were mostly highly outcrossing, and outcrossed nuts often had higher quality than selfed nuts. Growers should consider interplanting different cultivars more closely and distributing bee hives more widely to maximise cross-pollination, produce high yields, and optimise nut quality.

Keywords: breeding system; cross-pollination; *Macadamia integrifolia*; *Macadamia tetraphylla*; mating system; pollen limitation; pollination; Proteaceae; self-incompatibility; xenia

1. Introduction

Fruit and nut crops account for about 8% of global food production [1], and many of these crops are pollinated by animals [2–5]. Fruit and nut yields may be affected by global declines in wild pollinator populations and by shortfalls in the number of managed beehives needed to sustain crop pollination [6–10]. Inadequate pollinator populations are affecting plant reproductive output by reducing the quantity or quality of pollen deposited on the stigmas of flowers [11]. Pollen limitation appears to be most prevalent among self-incompatible species, bee-pollinated species, and tropical species [11–13]. No empirical evidence had been available previously to conclusively establish pollen limitation of whole-plant reproductive output in tree species [14,15]. However, we demonstrated recently that pollen limitation of fruit production occurs in cultivated trees of the mass-flowering subtropical species, *Macadamia integrifolia* [16]. This species, and its hybrids with *M. tetraphylla*, are cultivated in many countries to produce fruit that contain the edible macadamia kernel [17].

Macadamia flowers are pollinated mainly by bees [18–22]. The flowers are considered partially self-incompatible, with greater pollen tube growth and initial fruitlet set following cross-pollination than self-pollination [23–27]. Macadamia trees are propagated clonally by grafting [28,29], and orchards are often established with wide blocks that comprise multiple rows of a single clonal cultivar [30,31]. This planting design allows for irrigation, nutrient, pest, disease and harvest management to be tailored to each cultivar in the orchard, but it reduces the opportunities for cross-pollination (i.e., by another cultivar) in the middle of each single-cultivar block [16,30,31]. We have identified that most initially set fruitlets in the middle of a block of cultivar '816' trees arise from self-pollination, i.e., by selfing [32]. However, most of the selfed fruitlets abscise from the tree during the period of premature fruitlet drop, about six weeks after flowering [32]. As a result, almost all of the mature fruit of this cultivar at 26 weeks after flowering arise from cross-pollination; i.e., the realised mating system is highly outcrossing [31–33]. Very high levels of outcrossing among mature macadamia fruit have also been identified in commercial orchards of cultivars 'A4', 'A16' and 'Daddow' [16,30,31,34] and among single trees in a multi-cultivar research trial [35]. Approximately 20 cultivars are widely planted in macadamia orchards globally, and yet we know very little about the extent of outcrossing in most of these cultivars. Most macadamia cultivars, like most almond cultivars [36,37], may be effectively self-sterile, being highly dependent on outcrossing to produce mature fruit.

The outcrossed fruit of macadamia cultivar '816' have been shown to possess higher nut-in-shell (NIS) mass, kernel mass and kernel recovery (i.e., the percentage of NIS mass that is comprised of kernel mass) than the few selfed fruit remaining at nut maturity [16,31,32]. Pollen-parent effects on fruit characteristics are termed 'xenia' [38], and similar xenia effects have been observed in almond and hazelnut fruit [36,37,39]. Cross-pollination of macadamia flowers might be required not only for high yields, but also for maximal nut quality.

In this study, we aimed to determine whether high levels of outcrossing are common across a wide range of international macadamia cultivars in three countries. We assessed, wherever possible, the levels of outcrossing in the middle of a block of each single clonal cultivar, where the opportunities for cross-pollen transfer are likely to be lowest [16,30,31]. Furthermore, we aimed to determine whether outcrossed macadamia fruit often have higher nut mass, kernel mass, kernel recovery, and kernel oil concentration than selfed fruit. The results will help to identify the cross-pollination requirements of macadamia cultivars globally. This will assist growers to design orchards and manage pollinators in ways that ensure high yield and optimise nut quality.

2. Materials and Methods

2.1. Sample Sites and Processing

We sampled macadamia fruit from 19 cultivars at 23 sites in commercial orchards in Australia, Brazil and South Africa (Table 1). We sampled fruit of cultivars '814', '816' and 'A4' from orchards in both Australia and South Africa. We sampled fruit of cultivar '344' from one orchard in each of the main Australian production regions, i.e., southern Queensland and northern New South Wales. In each orchard, ten fruit were sampled from each of six trees per cultivar, providing a total of 60 fruit per site × 23 sites = 1380 fruit. Wherever possible, we selected trees in the middle row of a single-cultivar block. However, trees of cultivars '246' and 'H2' were in single rows that were located only one row away in each direction from another cultivar. Trees of cultivars '344' (at one site) and 'MCT1' were mixed in the same row with other cultivars. We usually sampled fruit from the 10th, 20th, 30th, 40th, 50th and 60th tree from the end of the row, although the tree separation was lower in orchards that had shorter rows (Table 1). The nearest cultivar in each direction, and all other cultivars within 500 m of the sampled trees, were recorded (Table 1).

The sampling method for each orchard reflected the commercial harvesting practices employed in each country. Fruit were sampled randomly from the orchard floor in Australia and Brazil. Fruit were sampled randomly from the tree canopy in South Africa, except that cultivar '814' fruit were sampled from the orchard floor. We sampled and dehusked fruit during the peak harvesting period for each orchard. Australian nuts-in-shell were then dried at 37 °C for 2 d, 45 °C for 2 d, and 57 °C for 2 d [40]. Brazilian nuts-in-shell were dried at room temperature for 3 months. Each nut-in-shell (NIS) was weighed, cracked manually, and its kernel was weighed (Table A1). South African nuts-in-shell were dried at 35 °C for 3 d and then at 45 °C until reaching constant mass. Each South African NIS was cracked manually, and the kernel was dried at 50 °C until reaching constant mass. We calculated the kernel recovery of each nut; i.e., the percentage of NIS mass that was comprised of kernel mass (Table A1). We also determined the oil concentration of each Australian kernel (Table A1) by measuring the specific gravity of a subsample of kernel that was placed on a pan immersed in 95% (v/v) aqueous ethanol [41]:

$$O_k\ (\%) = 284.7 - 212.57 \times G_s \quad (1)$$

where O_k was the kernel oil concentration and G_s was the specific gravity, and

$$G_s = (0.7995 \times M_a)/(M_a - M_e) \quad (2)$$

where M_a was the mass in air and M_e was the mass in 95% ethanol.

2.2. Kernel Genotyping

A crushed subsample of at least 30 mg of each kernel was used to determine its paternity. We extracted DNA following the glass-fibre plate DNA protocol for plants [42], using disposable 2.3 mm and 0.1 mm zirconia/silica beads prior to shaking on an MM2000 TissueLyser II (Retsch, Haan, Germany). We assigned kernel paternity in cultivars '741', '814', '816', '842', '849', 'A4', 'A16', 'A38', 'A203', 'Daddow' and 'Own Venture' from Australian orchards by high-throughput genotyping using the Agena MassARRAY platform (Agena Bioscience, San Diego, CA, USA). MassARRAY identifies single nucleotide polymorphisms (SNPs) that are both homozygous and unique to each macadamia cultivar, and so can be used to definitively identify the pollen-parent cultivar of each kernel. Details of the SNP markers and MassARRAY method used to identify macadamia pollen parents have been provided previously [16]. We amplified the extracted kernel DNA (2 µL; ~10 ng/µL) in 5 µL multiplex PCR reactions containing 1 U of Taq, 2.5 pmol of each PCR primer, and 500 µM of each dNTP (PCR Accessory and Enzyme Kit, Agena). We performed thermocycling at 94 °C for 4 min followed by 45 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 3 min. Unincorporated dNTPs were deactivated using 0.5 U of shrimp alkaline phosphatase (37 °C for 4 min, 85 °C for 5 min).

Table 1. Macadamia cultivar, orchard location, sample-tree separation within the row, distance to nearest other cultivars, nearest other cultivar in each direction, and other cultivars within 500 m.

Cultivar	Country	Location	Tree Separation within Row	Distance to Nearest Other Cultivars	Nearest Cultivar in Each Direction	Other Cultivars within 500 m
246	Australia	28°43′39″ S 153°24′08″ E	10 (50 m)	1 and 1 row (10 m and 10 m)	741, H2	333, 344, 508
344 [1]	Australia	24°58′36″ S 152°22′45″ E	10 (40 m)	6 and 6 rows (48 m and 48 m)	Daddow, A203	741, 842, 849, A4, A29, A268
344 [2]	Australia	28°44′29″ S 153°31′34″ E	10 (35 m)	Mixed in the same row (3.5 m)	246, 816, 849	333, 508, 741, 788, 791, A4
741	Australia	24°56′18″ S 152°21′38″ E	10 (20 m)	3 and 3 rows (30 m and 30 m)	A4, A203	814, 816, 842, 849, A16, A38, A268, Own Venture
814	Australia	24°55′45″ S 152°21′51″ E	5 (20 m)	3 and 3 rows (24 m and 24 m)	816, A16	741, 849, 842, A4, A38, A203, A268, Daddow, Own Venture
816	Australia	24°58′50″ S 152°22′46″ E	10 (40 m)	4 and 4 rows (32 m and 32 m)	842, A4	344, 741, 849, A29, A203, A268, Daddow
842	Australia	24°58′46″ S 152°22′46″ E	10 (40 m)	10 and 11 rows (80 m and 88 m)	741, 816	344, 849, A4, A29, A203, A268, Daddow
849	Australia	24°58′55″ S 152°22′46″ E	10 (40 m)	7 and 8 rows (56 m and 64 m) [3]	A4, forest [3]	344, 741, 816, 842, A29, A203, A268, Daddow
A4	Australia	24°45′44″ S 152°15′55″ E	10 (40 m)	4 and 5 rows (32 m and 40 m)	849, A38	344, 842, A16, A268
A16	Australia	24°55′47″ S 152°21′52″ E	10 (40 m)	4 and 4 rows (32 m and 32 m)	814, A38	741, 816, 849, 842, A4, A203, A268, Daddow, Own Venture
A29	Australia	24°56′21″ S 152°21′38″ E	10 (20 m)	3 and 3 rows (30 m and 30 m)	A203, A203	741, 816, 842, 849, A4, A16, A38, A268, Daddow, Own Venture
A38	Australia	24°56′14″ S 152°21′58″ E	10 (40 m)	6 and 7 rows (48 m and 58 m)	A268, 842	741, 816, 849, A4, A16, A203, Daddow, Own Venture
A203	Australia	24°58′40″ S 152°22′46″ E	10 (40 m)	4 and 5 rows (32 m and 40 m)	344, 741	816, 842, 849, A4, A29, A268, Daddow
A268	Australia	24°55′5″ S 152°21′55″ E	10 (40 m)	8 and 8 rows (56 m and 56 m)	842, Daddow	741, 816, 849, A4, A16, A38, A203, Own Venture
Daddow	Australia	24°58′31″ S 152°22′45″ E	10 (40 m)	8 and 9 rows (64 m and 72 m)	849, 344	741, 816, 842, A4, A29, A203, A268
H2	Australia	28°43′47″ S 153°24′06″ E	10 (50 m)	1 and 1 row (10 m and 10 m)	246, 508	333, 344, 660, 741
MCT1	Australia	24°50′42″ S 152°17′40″ E	10 (40 m)	Mixed in the same row (4 m)	See next column	741, A4, A16, A38, Daddow, Heilscher
Own Venture	Australia	24°55′51″ S 152°21′52″ E	10 (40 m)	4 and 4 rows (32 m and 32 m)	A38, 849	741, 816, 842, A4, A16, A203, A268, Daddow
IAC 4-12B	Brazil	22°22′17″ S 48°26′38″W	5 (20 m)	4 and 5 rows (35 m and 50 m)	246, 246	344, 420, 741, 816, 1014
695 (Beaumont)	South Africa	25°48′36″ S 31°0′24″ E	5 (30 m)	17 trees [4] and 20 rows (102 m and 240 m)	788, 814	741, 816, A4
814	South Africa	25°48′33″ S 31°0′35″ E	5 (30 m)	8 and 7 rows (100 m and 84 m)	695, forest [3]	788, 816
816	South Africa	25°48′21″ S 31°0′3″ E	5 (30 m)	26 trees [4] (130 m)	741, 741	695, 788
A4	South Africa	25°38′55″ S 31°18′33″ E	4 (16 m)	3 and 4 rows (24 m and 32 m)	695, A16	344, 741, 788, 816, 842, 849, A38, A203, A268, Daddow, Nelmak 2

[1,2] Orchards 1 and 2 of cv. 344. [3] Cv. 849 (Australia) and cv. 814 (South Africa) adjoined natural forest at the orchard boundary. [4] Cvv. 695 and 816 (South Africa) had a block of cv. 788 trees or cv. 741 trees at the end of their respective rows.

We assigned paternity to kernels from Brazilian and South African orchards, and from cultivars '246', '344', 'A29', 'A268', 'H2' and 'MCT1' in Australian orchards, using a genotype-by-sequencing (GBS) approach that identified the same SNPs that were identified in other cultivars by MassARRAY. The GBS approach was used for those Australian sites that possessed a complex mixture of potential pollen parents, including cultivars that were closely related to each other. The GBS library preparation followed a ddRAD protocol [43] with minor modifications. All samples were normalised to 5 ng/µL prior to input into the protocol. Normalised DNA was digested with a restriction enzyme pair of PstI and NlaIII (NEB), and barcoded adapters (IDT) were then ligated to the cohesive ends. We then pooled 48 uniquely barcoded, digested and ligated samples to form a library. Size selection was performed on libraries using the Blue Pippin platform (Sage Science, Beverley, MA, USA), selecting for fragments between 280 and 375 bp. Secondary indexing used uniquely indexed PP7 and non-indexed PP5 primers (IDT) in a PCR. The reaction conditions were 98 °C for 90 s plus 11 cycles of 98 °C for 10 s, 63 °C for 20 s, and 72 °C for 20 s. The final extension was performed at 72 °C for 7 min. Following a clean-up with SPRI-select beads (Beckman, Brea, CA, USA), libraries were sequenced as 150-bp paired end reads on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA), using an SP300 flow cell. The genotyping data were generated using Stacks software version 2.60. Briefly, the software took sequences in FASTQ.GZ format as an input and de-convoluted each read according to the inline barcodes. The pipeline also checked for read quality and restriction site presence. It created a separate FASTQ file for each sample. It then automatically trimmed FASTQ files to the size of the shortest read minus two bases to compensate for differences in read length due to any variation in barcode sequences. The alignment process was then launched. This process created stacks of similar reads for each sample individually, with these reads stacks also known as tags. The tags which appeared across all samples were then collated (catalogue tags), and genotypes (cultivars) were calculated for the common polymorphic sites.

2.3. Data Analysis

We identified the main pollen parents for each cultivar at each site and calculated the percentages of kernels that were cross-pollinated. The main pollen parents at each site were defined as any cultivar(s) that fathered at least six of the 60 fruit (i.e., 10% of the fruit) for each mother cultivar. We compared the effects of these main pollen parents on NIS mass, kernel mass, kernel recovery and kernel oil concentration using analyses of variance (ANOVA). Post-hoc Tukey's HSD tests were performed when ANOVA detected significant differences among more than two pollen parents. Means were regarded as significantly different at $p < 0.05$. Means are reported with standard errors.

3. Results

3.1. Outcrossing Levels

Most macadamia cultivars in Australian orchards were highly outcrossing, producing 80–100% of their kernels by cross-pollination rather than self-pollination (Figure 1). The lowest outcrossing levels in Australia were in cultivar '344' at one of two sites (48 ± 8%) and in cultivars '246' and 'A29' (62 ± 7% and 72 ± 8%, respectively).

Cultivar 'IAC 4-12B' was highly outcrossing in Brazil, producing 88 ± 2% of its kernels by cross-pollination (Figure 1). Cultivar 'A4' was highly outcrossing in South Africa, as it was in Australia. Cultivars '695' and '816' in South Africa produced 64 ± 8% and 41 ± 5%, respectively, of their kernels by cross-pollination. Lowest levels of outcrossing (10 ± 6%) were found among cultivar '814' kernels in South Africa.

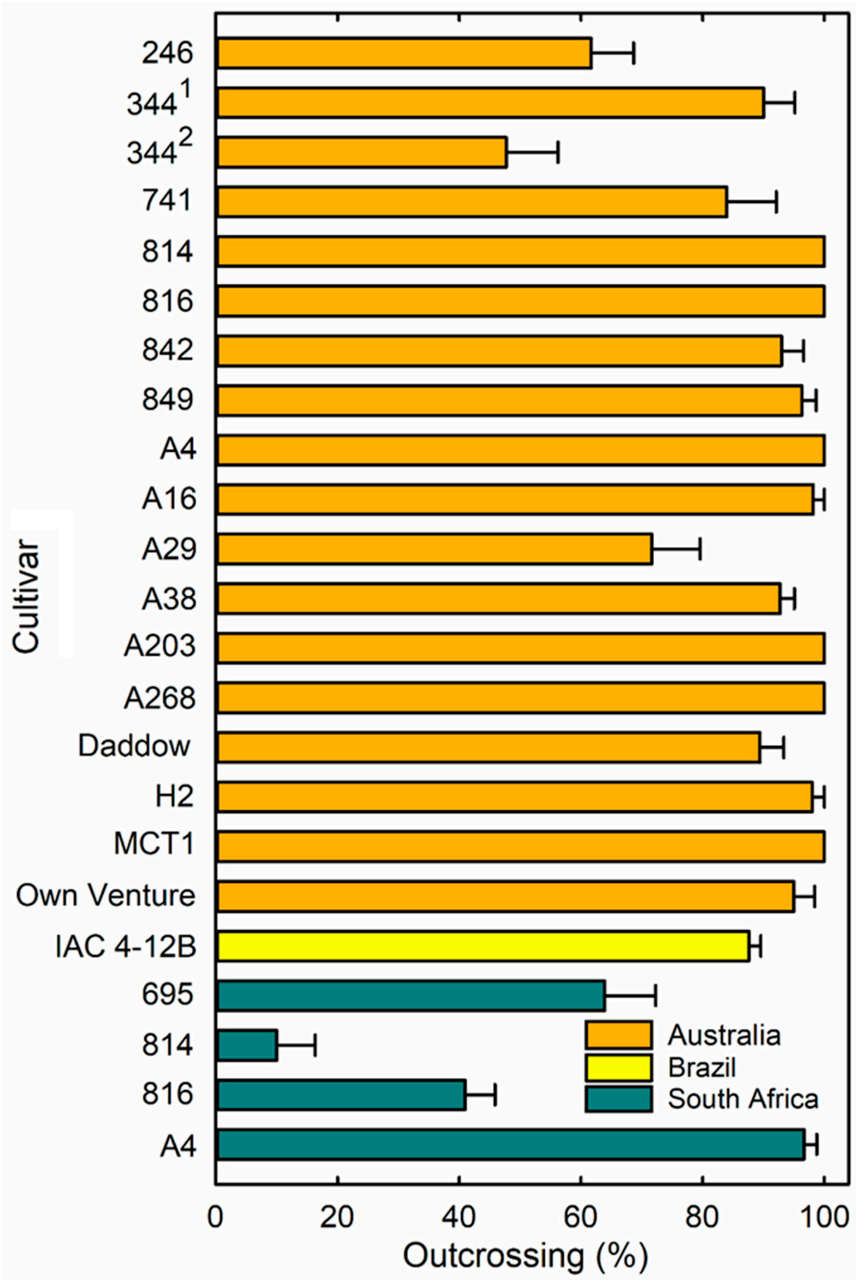

Figure 1. Mean (+SE) outcrossing levels among fruit of 19 macadamia cultivars from 23 sites in commercial orchards in Australia, Brazil and South Africa (n = six mother trees). 344 [1] and 344 [2] refer to Australian orchards 1 and 2, respectively, of cv. '344'.

The predominant cross-pollen parents in most orchards, denoted by an asterisk '*' (Table 2), were one or both of the nearest other cultivars (Table 1). However, other cultivars within 500 m (Table 1) were the predominant cross-pollen parents at the '695', '741', '842' and '849' sites (Table 2). The predominant pollen parent at the 'A29' site was 'H2' seedling rootstock (Table 2). The pollen parent of the 'H2' seedling rootstocks (i.e., the grandfather of the fruit) could be identified as mostly '741' although some of the 'H2' seedling rootstocks were fathered by '816', '835' or '849'.

Table 2. Nut-in-shell mass, kernel mass, kernel recovery and kernel oil concentration of macadamia fruit with different pollen parents. Pollen parents that provided at least six fruit (≥10% of fruit) are shown for each mother cultivar. The predominant cross-pollen parents for each site are indicated by asterisks (*).

Mother Cultivar × Pollen Parent	Country	Nut Size or Quality Parameter			
		Nut-in-Shell Mass (g)	Kernel Mass (g)	Kernel Recovery (%)	Oil Concentration (%)
246 × 246	Australia	7.21 ± 0.25 a	2.23 ± 0.11 a	30.4 ± 0.9 a	74.6 ± 0.6 a
246 × 344	Australia	7.63 ± 0.33 a	2.56 ± 0.13 ab	33.6 ± 1.1 ab	74.8 ± 0.7 a
246 × 508	Australia	8.12 ± 0.19 a	2.86 ± 0.12 b	35.2 ± 1.3 b	76.2 ± 1.1 a
246 × 741 *	Australia	6.80 ± 0.29 a	2.31 ± 0.10 ab	34.3 ± 1.0 b	76.8 ± 0.4 a
344 [1] × 344	Australia	6.79 ± 0.74 a	1.87 ± 0.33 a	26.4 ± 2.6 a	75.4 ± 1.5 a
344 [1] × 842	Australia	8.58 ± 0.31 b	2.70 ± 0.14 b	31.4 ± 0.7 ab	77.9 ± 0.6 ab
344 [1] × 849	Australia	8.90 ± 0.24 b	2.72 ± 0.11 b	30.6 ± 0.8 ab	77.0 ± 0.8 ab
344 [1] × Daddow *	Australia	8.40 ± 0.26 b	2.77 ± 0.12 b	32.8 ± 0.9 b	78.5 ± 0.5 b
344 [2] × 344	Australia	6.31 ± 0.34 a	1.69 ± 0.15 a	25.4 ± 1.3 a	73.5 ± 0.5 a
344 [2] × 246 *	Australia	8.35 ± 0.64 b	2.61 ± 0.23 b	31.2 ± 1.3 b	77.0 ± 0.9 b
741 × 741 *	Australia	5.36 ± 0.32 a	1.91 ± 0.10 a	36.0 ± 1.3 a	75.7 ± 0.8 a
741 × 842 *	Australia	7.12 ± 0.24 b	2.63 ± 0.12 b	36.9 ± 0.8 a	77.7 ± 0.4 ab
741 × A16	Australia	8.75 ± 0.32 c	3.46 ± 0.09 c	39.7 ± 1.4 a	79.2 ± 0.3 b
814 × 816 *	Australia	5.75 ± 0.25 a	1.96 ± 0.11 a	33.5 ± 1.1 a	77.6 ± 0.3 a
814 × 849 *	Australia	6.47 ± 0.26 ab	2.47 ± 0.10 b	38.3 ± 1.3 b	78.2 ± 0.4 a
814 × A16	Australia	7.57 ± 0.54 b	3.01 ± 0.18 c	38.8 ± 1.6 b	80.0 ± 0.6 b
816 × 842 *	Australia	6.91 ± 0.25 a	2.94 ± 0.11 a	42.7 ± 0.9 a	77.0 ± 0.4 a
816 × 849	Australia	6.97 ± 0.24 a	3.01 ± 0.10 a	43.3 ± 1.0 a	77.3 ± 0.4 a
842 × 816	Australia	6.91 ± 0.26 a	2.63 ± 0.12 a	38.2 ± 1.3 a	78.9 ± 0.4 a
842 × Daddow *	Australia	8.10 ± 0.32 b	3.28 ± 0.11 b	40.8 ± 1.2 a	79.2 ± 0.3 a
849 × 816 *	Australia	6.02 ± 0.23 a	2.54 ± 0.14 a	41.6 ± 1.4 a	76.9 ± 0.6 a
A4 × 344	Australia	6.95 ± 0.40 a	3.23 ± 0.17 a	46.6 ± 1.1 a	79.7 ± 0.5 ab
A4 × A16	Australia	8.03 ± 0.36 ab	3.69 ± 0.19 ab	45.9 ± 0.8 a	79.0 ± 0.6 a
A4 × A38 *	Australia	8.40 ± 0.29 b	4.01 ± 0.14 b	47.9 ± 1.1 a	80.7 ± 0.4 b
A16 × 814 *	Australia	7.85 ± 0.43 a	3.16 ± 0.20 a	40.2 ± 1.1 a	79.5 ± 0.8 a
A16 × A38 *	Australia	7.74 ± 0.40 a	3.32 ± 0.21 a	42.6 ± 0.9 a	78.8 ± 0.4 a
A29 × A29	Australia	8.26 ± 0.40 a	2.95 ± 0.19 a	35.4 ± 1.0 a	75.5 ± 0.8 a
A29 × (H2 × 741) *	Australia	8.32 ± 0.44 a	3.14 ± 0.23 a	37.7 ± 1.5 a	78.5 ± 0.9 b
A203 × 344 *	Australia	8.74 ± 0.19 a	2.98 ± 0.11 a	34.1 ± 1.0 a	78.9 ± 0.6 a
A203 × Daddow *	Australia	10.26 ± 0.46 b	3.72 ± 0.28 b	36.0 ± 1.5 a	80.8 ± 1.0 a
A268 × 816	Australia	11.13 ± 0.78 a	3.95 ± 0.32 a	35.4 ± 0.7 a	77.6 ± 1.1 a
A268 × 842 *	Australia	10.12 ± 0.35 a	3.48 ± 0.17 a	34.1 ± 0.8 a	78.1 ± 0.3 a
A268 × 849	Australia	10.01 ± 0.61 a	3.56 ± 0.33 a	35.1 ± 1.4 a	76.8 ± 0.5 a
Daddow × Daddow	Australia	8.40 ± 0.70 a	3.01 ± 0.28 a	35.7 ± 0.9 a	78.6 ± 0.2 ab
Daddow × 344	Australia	7.94 ± 0.47 a	3.30 ± 0.20 a	41.7 ± 1.5 b	79.7 ± 0.4 a
Daddow × 849 *	Australia	8.91 ± 0.48 a	3.45 ± 0.15 a	39.0 ± 0.6 b	77.7 ± 0.5 b
H2 × 508 *	Australia	9.84 ± 0.49 a	2.98 ± 0.13 a	30.6 ± 1.1 a	77.4 ± 0.3 a
MCT1 × A4 *	Australia	7.32 ± 0.24 a	3.33 ± 0.12 a	45.8 ± 1.1 a	77.0 ± 0.5 a
MCT1 × A16	Australia	6.62 ± 0.35 a	3.33 ± 0.20 a	50.3 ± 1.6 a	75.8 ± 0.8 a
Own Venture × 849 *	Australia	8.71 ± 0.46 a	3.50 ± 0.22 a	40.1 ± 1.2 a	76.0 ± 0.4 a
IAC 4-12 B × IAC 4-12 B	Brazil	5.41 ± 0.55 a	1.69 ± 0.14 a	31.7 ± 1.4 a	—
IAC 4-12 B × 246 *	Brazil	5.35 ± 0.16 a	1.82 ± 0.06 a	34.1 ± 0.8 a	—
695 × 695	South Africa	5.05 ± 0.16 a	1.96 ± 0.07 a	38.8 ± 0.8 a	—
695 × 788	South Africa	5.63 ± 0.26 a	2.49 ± 0.15 b	44.0 ± 1.1 b	—
695 × 814	South Africa	5.67 ± 0.39 a	2.39 ± 0.17 ab	42.2 ± 1.1 b	—
695 × 816 *	South Africa	4.99 ± 0.17 a	2.10 ± 0.09 ab	42.1 ± 0.9 b	—
814 × 814	South Africa	4.45 ± 0.10 a	1.67 ± 0.05 a	37.4 ± 0.6 a	—

Table 2. *Cont.*

Mother Cultivar × Pollen Parent	Country	Nut Size or Quality Parameter			
		Nut-in-Shell Mass (g)	Kernel Mass (g)	Kernel Recovery (%)	Oil Concentration (%)
816 × 816	South Africa	5.52 ± 0.23 a	2.74 ± 0.13 a	49.6 ± 0.9 a	—
816 × 695 *	South Africa	6.33 ± 0.45 a	3.35 ± 0.30 a	52.4 ± 1.7 a	—
816 × 741 *	South Africa	5.57 ± 0.29 a	2.95 ± 0.19 a	52.8 ± 1.5 a	—
A4 × 695 *	South Africa	6.91 ± 0.19 a	3.58 ± 0.12 a	51.5 ± 0.8 a	—
A4 × A268	South Africa	7.44 ± 0.64 a	3.69 ± 0.34 a	49.7 ± 1.5 a	—

[1,2] Orchards 1 and 2, respectively, of cultivar 344. Means ± SE with different letters within a mother cultivar at one site are significantly different (ANOVA, with Tukey's HSD test for >2 means; $p < 0.05$; n = 6–54 fruit).

3.2. Pollen-Parent Effects on Nut Quality

Most cultivars were highly outcrossing, but sufficient levels of selfing were detected in eight of the 19 cultivars to allow comparisons of quality between cross-pollinated and self-pollinated fruit (Table 2). Outcrossed fruit had significantly heavier NIS and kernels than self-pollinated fruit in cultivars '344' and '741', regardless of the cross-pollen parent. Cross-pollinated '246' and '695' fruit sometimes had significantly heavier kernels than self-pollinated fruit, depending on the cross-pollen parent. Cross-pollinated '816', 'A29', 'Daddow' and 'IAC 4-12B' fruit did not differ significantly in either NIS or kernel mass from self-pollinated fruit.

Cross-pollinated '246', '344', '695' and 'Daddow' fruit often had significantly higher kernel recovery than self-pollinated fruit, with the effects sometimes depending on the cross-pollen parent (Table 2). In addition, cross-pollinated '344', '741' and 'A29' fruit sometimes had higher oil concentration than self-pollinated fruit (Table 2).

Nut quality also differed significantly among some cross-pollen parents (Table 2). For example, NIS and kernel mass were highest in cultivars '741' or '814' when they were pollinated by 'A16', in cultivars '842' or 'A203' when they were pollinated by 'Daddow', and in cultivar 'A4' when it was pollinated by 'A38'.

4. Discussion

Our results show that high outcrossing (80–100%) was the realised mating system across a wide range of international macadamia cultivars, even in the middle of single-cultivar blocks where many of the flowers were likely to have been self-pollinated. This suggests that the flowers of most macadamia cultivars are highly dependent on cross-pollination to produce mature fruit. Macadamia yields can be lower in the middle of single-cultivar blocks than at the edges of the blocks where the trees are in closer proximity to the flowers of other cultivars [16,30]. These results together demonstrate that macadamia yields are constrained by a harmful combination of a highly-outcrossing mating system, long distances to a cross-pollen source, and limited dispersal of cross-pollen by pollinators across the orchards. About half of the honeybees in macadamia orchards may forage in the first row from their hive, with the other half dispersed up to 300 m from the hive but concentrated on trees with large floral displays [44]. Over half of the stingless bees may forage within the first two rows from their hive, with very few foraging more than 100 m from the hive [44]. Each macadamia tree can produce 100,000–400,000 flowers annually [16,45,46], but most flowering within Australian orchards is completed within a short period of 2–3 weeks in early spring [16,21,47,48]. The establishment of macadamia orchards with blocks that comprise multiple rows of a single mass-flowering cultivar may reduce the chances that bees travel between cultivars and deposit cross-pollen on flowers. The planting of multiple rows of each cultivar reduces the cost of some orchard management operations, but it can also lead to reduced yields due to inadequate cross-pollination [16,30,31]. Cross-pollen is sometimes dispersed effectively for only 30–40 m into single-cultivar blocks of avocado trees [49–52], with declining levels of outcrossing and lower yield deeper into the blocks [50–52]. The distance at which yields decline into single-cultivar macadamia blocks is not well understood, and so further research is needed to determine the optimal spatial designs and distances between cultivars that allow efficient macadamia orchard management while promoting high levels of cross-pollination.

The realised mating system of most macadamia cultivars was at least 80% outcrossing in the current study, but substantial percentages of selfed fruit were produced at some sites. Outcrossed nuts were often significantly larger than selfed nuts, with differences of 1.61–3.39 g in NIS mass, 0.53–1.55 g in kernel mass and 3.3–6.4% in kernel recovery. This demonstrates a xenia effect on fruit quality in a single-seeded fruit, as found previously in almond, hazelnut, lychee and mango [36,37,39,53,54]. Some macadamia cultivars, such as '741', have long been known to produce a mixture of large and small nuts [48,55]. The current results demonstrate that the large nuts tend to be outcrossed while the small nuts tend to be selfed. Macadamia growers are paid premiums for increasing kernel recovery, and macadamia processors receive higher prices for 'styles' of product that contain larger kernels [56–59]. Therefore, the presence of selfed nuts in an orchard can drive down financial returns to both growers and processors. For example, the differences in revenue to growers between selfed nuts and outcrossed nuts equate to USD 433–841 per ton based on prices of USD 3000 per ton of NIS at 33% kernel recovery, with a 3% premium paid for each additional 1% kernel recovery.

A very low level of outcrossing (10%) was found at just one site, i.e., the '814' site in South Africa. This site was unusual because the '814' trees were located at one end of an orchard, with bushland on three and a half sides of the '814' block. The only adjoining macadamia trees were in a block of cultivar '695' trees, which generally do not flower at the same time as '814' trees in South Africa [60]. Therefore, the opportunities for cross-pollen deposition may have been especially low at this site. The nuts in this block were extremely small, highlighting the negative consequences of cross-pollination failure for nut quality in macadamia orchards. A lack of cross-pollination is also likely to reduce tree yields, as very low yield has been reported in isolated orchard blocks that contain only one macadamia cultivar [61] and in the middle of very wide single-cultivar blocks [16,30].

Average oil concentrations were well above the 72% threshold required for 'Grade 1' kernels [62,63] and within the range of 76–80% typically seen for some of the same cultivars at other sites [16,41,64–66]. However, selfed kernels of cultivars '344', '741' and 'A29' had 3.0–3.5% lower oil concentrations than some outcrossed kernels. Our results show that the presence of selfed nuts can drive down not only kernel mass and kernel recovery, but also kernel oil concentration, and so it is important to maximise cross-pollination to ensure both high yield and optimal nut quality.

Nut quality sometimes also differed among fruit arising from different cross-pollen parents. Cultivars that produced large kernels as a mother tree also tended to produce large kernels when they were the pollen parent. The kernel is the embryo of the macadamia fruit [17], and so it was not surprising that the cross-pollen parent directly affected embryo development and kernel size. Differences in macadamia fruitlet growth between different cross-pollen parents have been observed as early as six weeks after pollination [67]. The results indicate that variations in nut quality in macadamia orchards can be attributed both to the presence of selfed fruit and to the presence of different cross-pollen parents among the outcrossed fruit. Strategic selection of macadamia cultivars as pollinisers could, therefore, be used to improve kernel quality and financial returns. The use of polliniser trees is becoming increasingly common in macadamia orchards, including (a) the planting of a polliniser tree in every third position in every third row, (b) the planting of single rows of polliniser trees in wide blocks of another cultivar, and (c) the replacement of damaged trees with polliniser trees of a different cultivar [16,68,69]. Planting a polliniser tree in every third position in every third row ensures that every tree in the orchard has a tree of another cultivar as at least one of its eight neighbouring trees [68]. We also envisage the possibility of planting polliniser trees around the perimeter of an orchard, perhaps focusing on positions where the bee hives are placed each year prior to flowering. Polliniser cultivars would need to have overlapping flowering times with the main cultivar to ensure cross-pollination [70–73]. If the polliniser trees are planted within the same rows as the main cultivar, they would ideally also have pest, disease, fertiliser, irrigation and harvest-time requirements similar to those of the main cultivar [74,75], as well as having similar nut quality to minimise variation within nut consignments delivered to the processor.

The predominant cross-pollen parent at most sites was one or both of the two cultivars in closest proximity to the mother trees, as found previously [16,30,31]. This again highlights that there is limited cross-pollen dispersal across macadamia orchards, so that cultivars may need to be interplanted more closely and bees may need to be distributed more widely across the orchard [75]. However, an interesting finding at one site was the large percentage of fruit that were fathered by the 'H2' seedlings that are used as grafting rootstocks. In addition, these rootstocks were identified as minor contributors to pollen parentage at several other sites. Clonal rootstocks are rarely used in macadamia orchards [17]. Instead, seedlings of cultivars 'H2' and '695' ('Beaumont') are the most commonly used macadamia rootstocks in Australia and South Africa, respectively [76]. Scion death, either in the nursery or the orchard, or the outgrowth of sucker shoots from rootstocks, ensures that rootstock flowers are a covert source of cross-pollen in some orchards. These rootstock flowers may sustain an underlying level of cross-pollination in the middle of wide blocks that were thought to contain flowers of only one cultivar. Four different cultivars were identified as fathers of the 'H2' seedlings used as grafting rootstocks at the 'A29' site in Australia. Four different cultivars were also identified as fathers of the '695' fruit in South Africa. These results demonstrate that tree-to-tree variation in macadamia orchards can be attributed partly to the wide genetic variability in the 'H2' and '695' seedlings, used as grafting rootstocks, that arises from having a wide array of pollen parents.

5. Conclusions

The realised mating system of international macadamia cultivars was mostly highly outcrossing, even in the middle of single-cultivar blocks where most flowers were likely to be self-pollinated and where yields are often subject to pollen limitation. The predominant pollen parents at most sites were one or both of the two nearest other cultivars. The results confirm observations that plant reproductive output is often dependent on the quantity or genotype of pollen deposited on the stigmas of flowers and that pollen limitation is most prevalent among self-incompatible, bee-pollinated, or tropical species. Outcrossed macadamia fruit often had higher nut quality than selfed fruit, demonstrating a xenia effect on fruit quality. The presence of selfed fruit in a macadamia orchard can drive down average kernel mass, kernel recovery and kernel oil concentration. Furthermore, some cross-pollen parents provided particularly high kernel mass and kernel recovery. Therefore, macadamia growers could interplant different cultivars more closely throughout orchards and strategically select polliniser cultivars to maximise cross-pollination, maintain high yields, optimise nut quality, and improve financial returns for both growers and processors.

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Appendix A

Table A1. Average nut-in-shell mass, kernel mass, kernel recovery and kernel oil concentration for each macadamia cultivar.

Cultivar	Country	Nut Size or Quality Parameter			
		Nut-in-Shell Mass (g)	Kernel Mass (g)	Kernel Recovery (%)	Oil Concentration (%)
246	Australia	7.73 ± 0.18	2.56 ± 0.08	32.7 ± 0.6	75.8 ± 0.3
344 [1]	Australia	8.07 ± 0.18	2.58 ± 0.08	31.6 ± 0.7	77.8 ± 0.3
344 [2]	Australia	6.81 ± 0.28	1.90 ± 0.12	26.5 ± 1.0	74.7 ± 0.5
741	Australia	6.85 ± 0.18	2.58 ± 0.08	37.6 ± 0.5	77.8 ± 0.3
814	Australia	6.36 ± 0.15	2.34 ± 0.07	36.4 ± 0.8	77.3 ± 0.3
816	Australia	6.90 ± 0.14	2.97 ± 0.07	43.1 ± 0.5	77.4 ± 0.2
842	Australia	6.92 ± 0.18	2.51 ± 0.09	36.0 ± 0.8	77.9 ± 0.3
849	Australia	6.31 ± 0.15	2.61 ± 0.08	41.1 ± 0.8	76.7 ± 0.3
A4	Australia	7.84 ± 0.17	3.58 ± 0.09	45.6 ± 0.5	76.7 ± 0.3
A16	Australia	7.71 ± 0.15	3.20 ± 0.09	41.2 ± 0.6	78.8 ± 0.2
A29	Australia	8.73 ± 0.19	3.26 ± 0.09	37.2 ± 0.5	77.0 ± 0.3
A38	Australia	6.37 ± 0.20	2.32 ± 0.10	35.7 ± 0.8	77.6 ± 0.5
A203	Australia	9.25 ± 0.26	3.21 ± 0.10	34.7 ± 0.4	79.3 ± 0.3
A268	Australia	10.53 ± 0.32	3.74 ± 0.13	35.3 ± 0.7	78.1 ± 1.9
Daddow	Australia	8.36 ± 0.21	3.23 ± 0.08	38.6 ± 0.4	78.1 ± 0.2
H2	Australia	8.74 ± 0.38	2.76 ± 0.13	31.7 ± 0.8	76.8 ± 0.3
MCT1	Australia	6.94 ± 0.18	3.30 ± 0.10	47.8 ± 0.8	76.9 ± 0.4
Own Venture	Australia	8.22 ± 0.20	3.07 ± 0.09	37.4 ± 0.5	76.8 ± 0.3
IAC 4-12B	Brazil	5.42 ± 0.11	1.81 ± 0.05	33.5 ± 0.6	—
695 (Beaumont)	South Africa	5.21 ± 0.10	2.15 ± 0.05	41.1 ± 0.5	—
814	South Africa	4.60 ± 0.11	1.76 ± 0.05	38.0 ± 0.6	—
816	South Africa	5.73 ± 0.17	2.91 ± 0.10	50.6 ± 0.7	—
A4	South Africa	6.90 ± 0.17	3.53 ± 0.10	50.9 ± 0.6	—

[1,2] Orchards 1 and 2 of cultivar 344. Means are presented with SEs (n = 36–60 fruit).

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