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Genetic relationships of macadamia cultivars and species revealed by AFLP markers

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Summary

World production of macadamia nuts is based on two species, the smooth shell *Macadamia integrifolia* Maiden and Betche, and the rough shell *Macadamia tetraphylla* L.A.S. Johnson, and their hybrids. One hundred and five AFLP markers were used to analyze 26 macadamia accessions representing four species: *M. integrifolia*, *M. tetraphylla*, *M. ternifolia*, and *M. hildebrandii* as well as a wild relative, *Hicksbeachia pinnatifolia* (rose nut). Each macadamia accession showed distinct AFLP fingerprints indicating a significant level of genetic variation in this macadamia germplasm collection. The four *Macadamia* species included in this study were clearly separated using cluster analysis with AFLP marker data. Based on a single accession, the separation of *M. ternifolia* from *M. integrifolia* suggested the relatively distant genetic relationship between these two species and casts doubts on the notion that the *M. ternifolia* may be a variant of *M. integrifolia*. Within the major cluster of *M. integrifolia*, nine established smooth shell cultivars were separated into two sub-clusters, suggesting the heterozygous nature of the original gene pool that had contributed to macadamia variety improvement programs. *M. hildebrandii* and *H. pinnatifolia* formed a distinct cluster and shared dramatically less genetic similarity with the other *Macadamia* species. Additional data would be needed to clarify the phylogenetic nature and status of *M. hildebrandii* in the genus *Macadamia*.

Introduction

Macadamia, of the family Proteaceae, originated in southeastern Queensland and northeastern New South Wales, Australia (Smith, 1976). Although nine species of macadamia are known to exist (Gross, 1995; McDonald & Ismail, 1995), edible nuts are obtained from only two species of the genus *Macadamia*: *M. integrifolia* Maiden and Betche, known as the smooth-shell type; and *M. tetraphylla* L.A.S. Johnson, commonly referred to as the rough shell type, and their hybrids. The first large scale commercial planting of macadamia as a crop started in Hawaii in 1922. The Hawaii Agriculture Experiment Station began a macadamia improvement program through the selection of superior seedlings from various sources in 1936. The first five varieties were released in 1948 after intensive selection on 20,000 seedlings from various sources at the Hawaii Agricultural Experiment Station (Storey, 1948). Macadamia became fully established as a commercial crop in Hawaii during the 1940s, and then subsequently in California, Australia, and other tropical and sub-tropical countries.

The macadamia industry in Hawaii is entirely based on the smooth shell type *M. integrifolia* with about a dozen cultivars. The rough shell varieties are erratic and slow to come into bearing in Hawaii, and they produce lower-grade nuts (Hamilton & Fukunaga, 1973). The rough shell and interspecific hybrid cultivars are better adapted to cultivation in California and Australia (Smith, 1976). The breeding program in Hawaii was based on open-pollinated progenies from *M. integrifolia* plants imported from Australia in 1882 and 1892 (Hamilton & Fukunaga, 1959). The original *M. integrifolia* plants produced many superior cultivars and valuable breeding lines currently

used in macadamia improvement programs worldwide (Aradhya et al., 1998).

Knowledge of the genetic distance between individuals is invaluable in breeding programs to avoid problems such as inbreeding depression and to maximize genetic diversity. This is especially true with macadamia, as global production relies upon a limited gene pool (Hamilton & Fukunaga, 1959; Shigeura & Ooka, 1984). Assessment of variability in macadamia germplasm formerly relied on differences in morphological and agronomic traits. Molecular marker techniques have recently been employed as tools useful in assessing germplasm diversity. Such techniques could be of great advantage in macadamia breeding programs due to the long generation time of fifteen years, since DNA markers can be used for selection at the seedling stage once the markers associated with the target traits were identified. Isozyme markers have been utilized in fingerprinting macadamia species and in assessing genetic diversity within macadamia cultivars (Vithanage & Winks, 1992; Aradhya et al., 1998). However, the small number of markers the technique generates and the low polymorphism rate among commercial cultivars limit isozyme technology. To overcome this, randomly amplified polymorphic DNA (RAPD) and sequence tagged sites (STSs) markers were used to assess the genetic diversity of macadamia (Vithanage & Hardner, 1998).

AFLP is a relatively new molecular marker technique with the capacity to reveal many polymorphic markers in a single reaction (Vos et al., 1995). Comparative studies have indicated that AFLP is the most efficient method to estimate genetic diversity because of its high reproducibility and multiplex ratio when compared with other techniques such as RFLP, RAPD, and SSR (Powell et al., 1996; Russell et al., 1997; Pejic et al., 1998). The reliability of AFLP markers has been tested through sequencing 20 of the 117 putatively homologous AFLP markers in potato, and only one (5%) was found non-homologous (Rouppe van der Voort et al., 1997). AFLP markers have been widely used to analyze genetic diversity in numerous plant species such as Arabidopsis (Breyne et al., 1999; Erschadi et al., 2000), maize (Lubberstedt et al., 2000), and rice (Mackill et al., 1996; Zhu et al., 1998). We present here the results of our study on the genetic diversity of macadamia cultivars and their related species using AFLP markers.

Materials and methods

Plant material

Twenty-six macadamia accessions representing four Macadamia species and one *Hicksbeachia* were used in the fingerprint analysis, including eighteen accessions of *M. integrifolia*, two accessions of *M. tetraphylla*, four accessions of interspecific hybrids, one accession each of *M. ternifolia*, *M. hildebrandii*, and *H. pinnatifolia* (Table 1). All leaf samples were obtained from trees grown and maintained at the USDA, ARS, Pacific Basin Agricultural Research Center, Tropical Plant Genetic Resource Management in Hilo, Hawaii.

DNA isolation

Young macadamia leaves were collected and lyophilized over a period of 2–3 days for DNA extraction. A modified version of the extraction protocol described by Chittenden et al. (1994) was followed. Lyophilized tissue was ground to a fine powder with a Udy sample mill (Udy Corp, Ft. Collins, CO, USA). Ground leaf tissue was added to a 50 mL centrifuge tube to the 7 mL mark and 30 mL of 65 °C extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 1.25% SDS, 2% PVP-40, and 27.5 mM $NaHSO_3$) was added. Samples were mixed thoroughly by vortexing and placed in a 65 °C water-bath for 1 h with periodic mixing. Nine mL of 5M KOAc was added to each tube. The tube was inverted several times and placed on ice for 20 min. Samples were then centrifuged at 2,800 g for 20 min at 4 °C. After centrifugation, the supernatant was removed from the cellular debris by filtering through Miracloth (Calbiochem) to a new tube containing 20 mL of ice-cold isopropanol (–20 °C). Samples were stored at –20 °C for 2 h. The DNA was then spooled out and placed in 1 mL of purifying buffer (70% ethanol, 0.3 M NaOAc) and stored at –20 °C overnight. After removing the purifying buffer, the pellets were rinsed in 70% ethanol, air dried, and resuspended in 300–500 μL of TE. RNA was removed with 50 μg of RNAse A and incubated at 37 °C for 30 min. A phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by a chloroform:isoamyl alcohol (24:1) extraction achieved further purification. Two volumes of 95% ethanol and 1/25 volume of 5M NaCl were added to the extracted DNA then incubated at –20 °C for 30 min and centrifuged at 15,300 g for 15 min. The pellets were rinsed in 70% ethanol, air-dried, and

Table 1. List of Macadamia varieties and related species analyzed by ALFP markers

Acc. No.	Variety	Origin
M. integrifolia		
HMAC1	Kau, HAES 344	Selected in 1971 at the Nutridge Orchard of the Hawaii Macadamia Nut Co., Honolulu, Oahu
HMAC2	Keaau, HAES 660	Selected in 1966 at the Deschwanden Orchard, Lawai Valley, Kauai
HMAC3	Keauhou, HAES 246	Selected in 1948 at the Keauhou Orchard of Hawaii Macadamia Nut Co. at Kona, Hawaii
HMAC4	Makai, HAES 800	Selected in 1977 from open pollinated progeny of 'Keauhou' at the Waiakea Expt. Station, Hawaii
HMAC5	Mauka, HAES 741	Selected in 1957 at the Glaisyer Orchard, Lawai Valley, Kauai
HMAC6	Pahala, HAES 788	Selected in 1981 from open pollinated progeny of 'Keauhou' at the Poamoho Expt. Station, Oahu
HMAC7	Purvis, HAES 294	Selected in 1981 at the Nutridge Orchard of the Hawaii Macadamia Nut Co., Honolulu, Oahu
HMAC8	Ikaika, HAES 333	Selected in 1952 at the Nutridge Orchard of the Hawaii Macadamia Nut Co., Honolulu, Oahu
HMAC9	Kakea, HAES 508	Selected in 1948 at the Nutridge Orchard of the Hawaii Macadamia Nut Co., Honolulu, Oahu
HMAC10	Faulkner	California
HMAC23	McCormack	
HMAC24	N90-32	
HMAC33	N95-43	Thailand, Kau Kor #1, an open pollinated seedling selection, Dept. of Ag, Hort. Res. Station, Bangkok
HMAC32	N95-42	Thailand, Kau Kor #3, an open pollinated seedling selection, Ibid.
HMAC34	N95-24	An open pollinated seedling selected at Waiwi, Thailand.
HMAC39	Select X 344	
HMAC40	Select-16	
HMAC41	Select-59 (790)	
M. tetraphylla		
HMAC14	Burdick	California
HMAC17	Probert 2	Australia
Macadamia hybrid *		
HMAC18	Beaumont	Selected in 1954 at the Goswell property at Highfields, Australia
HMAC19	A16	Selected from open pollinated progeny of 'Renown' in Australia
HMAC21	Greber	Selected in 1948 from Greber's property at Amamoore in Australia
HMAC22	Probert 1	
M. ternifolia		
HMAC36		
M. hildebrandii		
HMAC35		Sulawesi, Indonesia
Hicksbeachia pinnatifolia		
HMAC37		Australia

* Originated from unassisted hybridizations between *M. integrifolia* and *M. tetraphylla*.

resuspended in TE. DNA concentration was estimated by comparison to serial dilutions of a lambda DNA standard in a 1.0% agarose gel.

AFLP analysis

Genomic DNA Digestion: AFLP reactions were performed according to the protocol of Vos et al. (1995) with the modification that 250 ng of DNA were digested at 37 °C for 3 h with 5U each of *Eco*R I and *Mse* I.

Adapter ligation and Pre-amplification: Adapters and primers were synthesized by Operon Technologies. Preamplification was performed as described by Vos et al. (1995) except that 1 unit of Taq polymerase (Promega) was used.

Primer Labeling and Selective Amplification: *Eco*R I and *Mse* I primers containing three selective nucleotides were obtained from Life Technologies. Reactions were performed according to the manual in the AFLP Analysis System I (Cat No. 10544-013, Life Technologies) except that gamma ^{32}P-ATP (6000Ci/mmol) was used. An initial screen of 32 primer combinations was performed, and those yielding banding patterns with good resolution and a high rate of polymorphism were selected for analysis.

Gel Analysis: To each PCR product was added 20 μL of formamide dye (98% formamide, 10 mM EDTA, 0.005% xylene cyanol FF, and 0.005% bromophenol blue), and the samples were denatured at 95 °C for 3 min. Three μL of sample was loaded onto a pre-warmed 5% polyacrylamide gel and run for 2.5 h at 105W. Gels were transferred to 3M Whatman paper and vacuum dried for 1–1.5 h at 80 °C, then exposed to X-ray film at 80 °C for 1–2 days with one intensifying screen.

Data analysis

Only polymorphic AFLP markers were scored, with presence as (1) and absence as (0). We assume that the same band represents the same allele in different accessions. The data were used to compute pairwise simple matching coefficients (Sokal & Michener, 1958). The resulting similarity matrix was used in cluster analysis, following the UPGMA algorithm (Sneath and Sokal 1973) provided in the software program NTSYSpc, version 2.1 (Exeter Software Co., New York). Goodness of fit was measured by calculating the correlation coefficient between the cophenetic and similarity matrices (Rohlf & Sokal, 1981). The cophenetic value matrix (matrix of ultrametric values) was derived from a tree matrix produced by the SAHN program that performs the sequential, agglomerative, hierarchical, and nested clustering methods as defined by Sneath & Sokal (1973).

Table 2. Primer pairs used in *Macadamia* fingerprinting

Primer pair	No. of polymorphic markers
E-AGG, M-CAA	20
E-AGG, M-CAG	10
E-AGG, M-CAT	18
E-ACC, M-CAT	20
E-ACC, M-CTA	10
E-AAC, M-CAA	27
Total	**105**

Results

Fifteen *Eco*R I – *Mse* I primer combinations (each containing three selective nucleotides) were surveyed with three accessions of *M. integrifolia* and one *M. tetraphylla* to assess variation detected by different primer sets. This allowed for selection of those primer pairs that generated the highest level of polymorphism for full-scale analysis. High levels of variation between the two species were found. The average number of polymorphic bands per primer set within the *M. integrifolia* selections was 14.6 with a range of 9 to 21. All of the primers screened generated a significant number of polymorphisms within the samples surveyed. Based on these results, six primer pairs were chosen for the final analysis, and 105 polymorphic bands were scored (Table 2) within the 27 accessions evaluated.

Genetic similarities based on simple matching coefficients revealed a significant level of diversity within the accessions evaluated (Table 3). The average genetic similarity among all 27 accessions evaluated was 0.742 with a range of 0.326 to 0.985. The average genetic similarity within the 18 *M. integrifolia* accessions was 0.839 and ranged from 0.697 to 0.985. *M. integrifolia* was found to be most genetically similar to *M. tetraphylla* (0.726) and the least similar to the wild relative *Hicksbeachia pinnatifolia* (0.369). *M. tetraphylla* was slightly more similar to *M. integrifolia* than to *M. ternifolia* (0.687), and *M. ternifolia* was almost equally similar to *M. integrifolia* (0.681) as it was to *M. tetraphylla* (0.687). Overall, similarity within the macadamia selections of four species was 0.774.

Table 3. Average genetic similarity based on SM coefficient within and between *Macadamia* species and interspecific hybrids

	M. integrifolia	*M. tetraphylla*	Hybrids	*M. ternifolia*	*M. hildebrandii*
M. integrifolia	0.839*				
M. tetraphylla	0.726	0.879*			
Hybrids	0.806	0.769	0.811*		
M. ternifolia	0.681	0.687	0.659		
M. hildebrandii	0.410	0.388	0.406	0.477	
H. pinnatifolia	0.369	0.426	0.356	0.402	0.587

* Average genetic similarity within species and hybrids based on multiple samples.

Genetic similarities generated from the Dice coefficient (Dice, 1945) were slightly higher than those obtained from the simple matching coefficient (data not shown). This caused one discrepancy in the two phenograms generated: the Dice coefficients implied that *M. integrifolia* was more similar to its wild relative, the rose nut, *H. pinnatifolia*, than to *M. hildebrandii*. Both methods produced matrices of high congruence, as determined by the co-phenetic correlation coefficients (SM, r = 0.982; Dice, r = 0.988).

The genetic relationships of macadamia cultivars and related species were further evaluated by cluster analysis using the UPGMA algorithm. Four major clusters were formed and each accession clustered according to species (Figure 1). All 18 *M. integrifolia* accessions plus three interspecific hybrids (Beaumont, Greber, and A16) clustered together at a similarity of approximately 81%. Within this group, five subclusters consisting of established Hawaiian cultivars were formed. The first included the cultivars Kau (Hamilton & Nakamura, 1971), Makai (Hamilton & Ito, 1977), and a hybrid, A16. The second subcluster included Keaau (Hamilton & Ooka, 1966), Mauka (Hamilton & Ito, 1977), Pahala (Hamilton et al., 1981a), and an unreleased selection, #16. Two of these Hawaiian selections, Keaau and Mauka, shared the highest genetic similarity of 0.985. The third sub-cluster of Hawaiian cultivars included Keauhou (Storey, 1948), Ikaika (Hamilton et al., 1952), Kakea (Storey, 1948), Purvis (Hamilton et al., 1981b), and an unreleased selection, #59. The fourth subcluster consisted of the two open pollinated seedlings, N95-42 and N95-24. The fifth subcluster consisted of three accessions (Faulkner, McCormack, and SelectX344) that have little documentation other than note that the Faulkner is from a California selection.

The final three major clusters comprised accessions from the other species evaluated. The two *M. tetraphylla* selections as well as Probert 1, an interspecific hybrid, formed a distinct cluster at a similarity of 0.820. Only one accession representing the species *M. ternifolia* was evaluated and it formed a separate cluster sharing a similarity of approximately 0.680 with *M. integrifolia* and *M. tetraphylla*. The final cluster included *M. hildebrandii* and a wild relative, *H. pinnatifolia* at a more distant similarity of 0.590. These two accessions were more closely related to each other than either was to the other three *Macadamia* species.

Discussion

The Hawaiian macadamia cultivars were selected from plant materials originally imported from their native Australia through independent introductions by W. Purvis of Kukuihaele, Hawaii, between 1882 and 1885, and by E.W. Jordan and R.A. Jordan of Honolulu, Oahu, in 1892 (Hamilton & Fukunaga, 1959; Shigeura & Ooka, 1984). The California and Australia macadamia industries were developed much later with selected varieties imported from Hawaii. It had been thought that macadamia cultivars and breeding materials were based on a small gene pool and that genetic diversity might be confined to this limited resource. However, we found that the degree of genetic variation among macadamia cultivars was much higher than that detected in coffee (Steiger et al., 2002) and papaya (Kim et al., 2002). The extent of variation in macadamia might be explained by the heterozygosity existing in the natural populations and the method used in the macadamia selection program (Hamilton & Fukunaga, 1959). The macadamia improvement programs in Hawaii were based on selection of seedlings of open-pollinated progenies. The selected trees were propagated through grafting to preserve the genotype

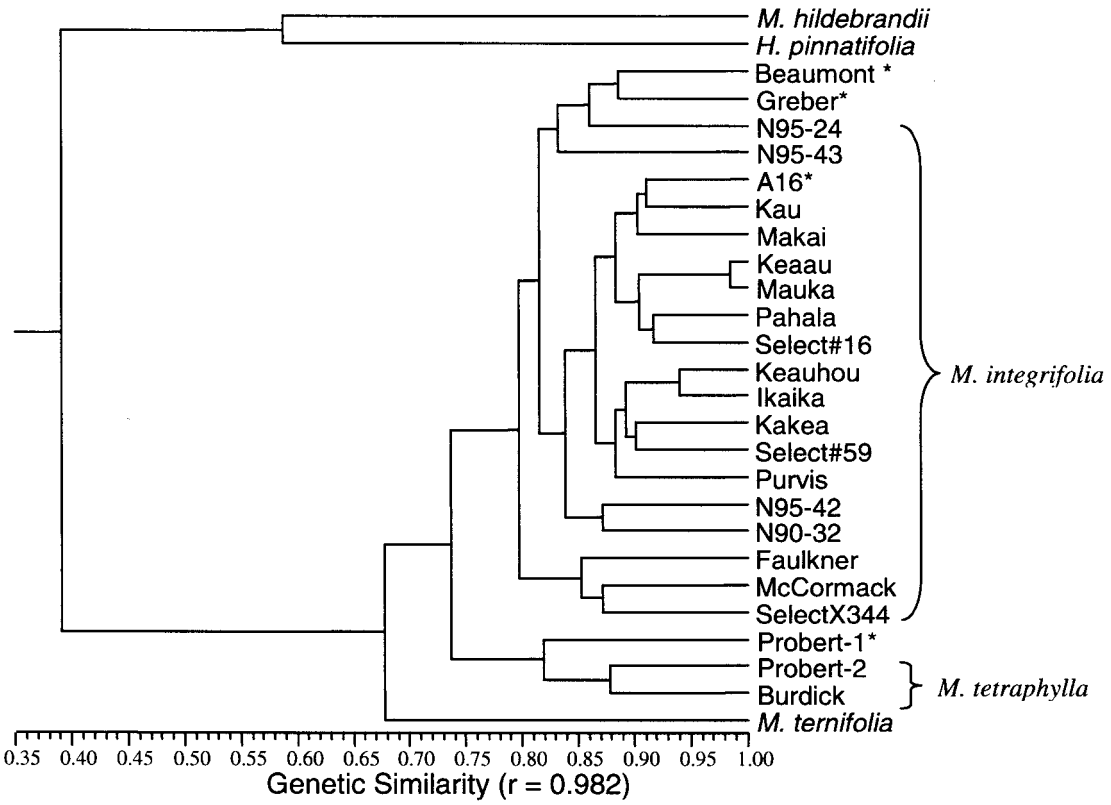

Figure 1. Phenogram based on simple matching coefficient of similarity among macadamia accessions and related species. Cophenetic correlation coefficient = 0.98. * Hybrid varieties (see Table 1).

since seed progenies of a cultivar always segregate (Storey & Hamilton, 1953; Hamilton & Fukunaga, 1962). The initial selection of Hawaiian cultivars was based on 20,000 seedlings in 1936 and up to 100,000 seedlings by 1976 from different orchards (Storey, 1948; Hamilton & Ito, 1976).

The nine established smooth shell cultivars were separated into two sub-clusters, but there is no clear evidence that these two sub-clusters corresponded to the two separate introductions by Purvis and Jordan. Based on historical records, the Purvis germplasm was from the Mt Bauple region (McConachie, 1980), while the Jordan germplasm was from the Hotham Creek / Pimpama region south of Brisbane, Australia (Wagner-Wright, 1995). These two regions are at the opposite ends of the native range of *M. integrifolia*. If the two sub-clusters of Hawaii cultivars reflect the two germplasm introductions, these two groups of cultivar would not be expected to be so closely related compared to the other three sub-clusters of the 18 *M. integrifolia* accessions. Moreover, the cultivar Makai was selected from open pollinated progeny of Keauhou at the Waiakea Experiment Station, Hawaii, but Makai and Keauhou were separated into different sub-clusters. It has been hypothesized that all Hawaiian cultivars were derived from the Jordan germplasm based on the following field observations: high degree of heterozygosity existed in the Jordan trees resulting in highly variable seedlings, while the Purvis trees were much more homozygous (Wagner-Wright, 1995); there were no cultivars derived from the Honakaa Sugar Company's orchard of purely Purvis tree seedlings (Wagner-Wright, 1995). The marginal differences of these two sub-clusters provided supporting evidence for this hypothesis.

Although there were some differences, the two sub-clusters of the nine established cultivars generated from AFLP markers mostly agreed with the clusters based on isozyme data (Aradhya et al., 1998). Three of the nine cultivars, Purvis, Kakea and Makai, grouped

in different sub-clusters based on isozymes versus AFLPs. Other differences include A16 in Aradhya's group 1c, Faulkner in 1e, and McCormack in 1a (Aradhya et al., 1998). These differences may be due to the nature of these two marker systems. Isozyme markers detect variation in expression of genes that tend to be conserved, while AFLP markers detect polymorphisms in both coding and non-coding regions of the genome.

Our initial survey of AFLP primers indicated abundant polymorphic markers in macadamia germplasm as compared to the amount found in papaya. The average number of polymorphic markers per primer set was 14.6 for macadamia and only 3.2 for papaya (Kim et al., 2002). Discrete fingerprints were found for each macadamia cultivar and accession examined. The DNA fingerprinting results also confirmed substantial genetic variation even within the cultivars of the smooth shelled species *M. integrifolia*. Eighteen *M. integrifolia* cultivars and accessions included in this study were spread across five sub-clusters (Figure 1).

Diversity in random genetic markers from random drift and mutation of DNA is proportional to the time since genotypes were separated. Morphological diversity is more likely a result of human selection and its relationship with fitness. For example, differences in productivity, nut and kernel characteristics, and tree forms were observed in several cultivars (Kau, Purvis, Ikaika, Kakea) selected from the same orchard (Storey, 1948; Hamilton et al., 1952). Our data for these cultivars showed sufficient genetic diversity that they were grouped to two sub-clusters. Other cultivars grouped on different sub-clusters, such as Kau and Keauhou, are very similar in nut characteristics, kernel quality, and productivity. However, Kau trees are upright and compact while Keauhou trees are more broad and spreading (Hamilton & Fukunaga, 1973). The cultivar Makai was selected from 4000 seedlings of open-pollinated Keauhou in 1967 and released in 1977. Makai mostly resembles Keauhou in tree form, nut characteristics, and yield potential, but kernel quality and percent of grade one kernels of Makai are significantly higher than those of Keauhou (Hamilton & Ito, 1977). Despite Makai being the direct descendant of Keauhou, these two cultivars were grouped into two separate sub-clusters. The two sub-clusters among established Hawaiian cultivars demonstrate the heterozygosity of the macadamia genome, which Ito et al. (1970) suggested that is partly a result of cross-pollination. The most closely related cultivars were Keaau and Mauka with genetic variation as little as 1.5% based on 105 AFLP markers. Keaau was first selected in 1948 and named in 1966 at the Deschwanden Orchard in Lawai Valley on Kauai (Hamilton & Ooka, 1966). It has an upright growing habit and outstanding nut and kernel characteristics. Mauka was first selected in 1957 and named in 1977 at the Glaisyer Orchard in Lawai Valley on Kauai (Hamilton & Ito, 1977). It also has an upright growing habit. The nut and kernel characteristics of Keaau and Mauka were very similar. However, Mauka performed substantially better than other cultivars at relatively high elevations from 1800 to 2200 feet (Hamilton & Ito, 1977). The close genetic relationship of these two cultivars hinted that they might share a common ancestor, although there is no record regarding their parental trees.

Previous isozyme data suggested considerable polymorphism among macadamia accessions (Vithanage & Winks, 1992; Aradhya et al., 1998). However, the isozyme polymorphism was not sufficient to separate the two Hawaiian varieties, Keauhou and Ikaika (Aradhya et al., 1998). AFLP markers generated from this project detected 7% genetic difference between these two cultivars. Keauhou is one of the oldest and best performing Hawaiian cultivars and was selected in 1935 and named in 1948 (Storey, 1948). It has excellent nut and kernel characteristics, but the tree is relatively broad and spreading. Ikaika was selected largely because of its tree vigor and hardiness for wind resistance, but its nut and kernel characteristics are not as desirable as other Hawaiian cultivars (Hamilton et al., 1952).

Although limited samples of other *Macadamia* species and interspecific hybrids were included in this survey, we were able to evaluate the relationship among them by cluster analysis. Two *M. tetraphylla* samples along with a hybrid formed a cluster sharing a genetic similarity of 0.727 with *M. integrifolia*. Three of the four hybrid samples grouped with *M. integrifolia* suggesting that more dominant markers were detected by AFLP in *M. integrifolia*. This confirms the high degree of homology between the genomes of *M. tetraphylla* and *M. integrifolia* as has been shown in complete chromosome paring in their hybrids (Storey & Saleeb, 1970). The *M. ternifolia* tree formed its own separate group. Interspecific hybrids between *M. integrifolia* and *M. tetraphylla* are reported to exist, supporting our grouping of *M. ternifolia* separately from *M. integrifolia*. The wild relative of macadamia, rose nut (*H. pinnatifolia)*, and *H. pinnatifolia* formed a distinct cluster and shared dramatically

less genetic similarity with other *Macadamia* species. More samples will be needed to access whether *M. hildebrandii* should continue to be classified as a species of *Macadamia*, considering the limited genetic similarity (0.416) between *M. hildebrandii* and other species of *Macadamia* revealed by AFLP markers.

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