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Hybrid Detection in Lychee (*Litchee chinensis* Sonn.) Cultivars Using HAT-RAPD Markers

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ABSTRACT: In this paper we present a method to detect hybrids obtained from open-pollinated seeds in lychee cultivars (*Litchi chinensis* Sonn.) using the high annealing temperature randomly amplified polymorphic DNA (HAT-RAPD) methodology. A series of 10 arbitrary random primers were used to amplify polymorphic DNA bands ranging from 200 to 1,700 bp. These bands were tracked from parent to hybrid offspring for crosses of four economically important lychee varieties (Chacrapat, KimJeng, Hong Huey and O-Hia) and both gender and variety specific band transmission rates were assessed. Irrespective of lychee variety, all hybrids showed a significant loss of parental bands with the largest losses occurring between the male parent and the offspring. Variety-specific bands were characterized for each lychee variety and hybrid band transmission rates were determined such that hybrids could be detected to any level of desired significance given enough initial banding data. This molecular marker technique provides a clear and compelling way to ascertain hybrid status of new plants without having to wait the six to seven years for the plants to mature.

KEYWORDS: Hybrid Detection; lychee cultivars; HAT-RAPD Markers; Chacrapat; KimJeng; Hong Huey and O-Hia.

INTRODUCTION

Lychee (*Litchi chinensis* Sonn.) is a tropical fruit with a white translucent aril, attractive red skin color, and sweet taste currently grown commercially in China, Thailand and Taiwan, with major markets for fresh fruit in Singapore, Hong Kong and Japan[1,2]. Other lychee-producing countries include India, Australia, South Africa, the Malagasy Republic, and the United States of America. In Thailand, lychee cultivars have long been grown in the northern and eastern parts of the country using both open-pollinated seeds and vegetative propagules[3]. One difficulty with such lychee propagation is due to the six to seven year lag before trees come to maturity, since crosses between different lychee varieties are often not true to type and upon reaching maturity may not bear edible fruit. Therefore the ability to detect hybrids before the trees come to maturity is of critical importance to lychee breeders, and it could present a significant increase in the rate of lychee selection if young plants could be rejected before waiting the six to seven years.

The genotypic identification of breeding lines, hybrids and clones is commonly based on morphological and anatomical features, but such phenotypic traits seldom serve as unambiguous descriptors since they are dependent on the environmental and physiological conditions of the plant[4]. To overcome this difficulty, alternative laboratory based methods, such as karyological measurement[5], chromatography[6], protein electrophoresis[7], and isozyme analysis have been proposed, but only isozyme analysis has proven to be a useful method for some levels of taxa determination[8]. Due to the limited amount of polymorphism found amongst closely related genotypes, even isozyme analysis has been found to have drawbacks characterizing closely related hybrids.

Molecular marker techniques overcome many of the limitations of morphological and biochemical techniques, since they are not affected by the environment or developmental stage and can detect variation at the DNA level[10]. For perennial crops like lychee where progress in crop improvement is hampered by its long generation period (six to seven years to maturity), the use of molecular markers is even more critical. To date, molecular markers have been used in a wide variety of applications, including: phylogenetic evaluation[11,12], molecular evolution[13], tagging and marker aided selection of economically important crop traits[14,15], and the mapping of quantitative trait loci (QTL) [16]. Some molecular techniques, such as DNA fingerprinting using mini- and micro-satellite DNA sequences and RFLP analysis[17], are potentially able to provide an unlimited number of

polymorphic markers, but they require knowledge of the targeted genome, require large amounts of DNA, are costly, labor intensive and commonly use radioactivity.

Another technique called RAPD-PCR uses randomly amplified polymorphic DNA to quickly scan an entire genome using single short arbitrary primers[18,19]. Since the abundance of priming sites for short primers follows a known statistical distribution, no prior knowledge of the genome is required. This allows previously uncharacterized genomes to be quickly and easily scanned for polymorphisms using a simple PCR based laboratory assay, which uses no radioactivity, and only requires a small amount of genomic DNA. When the RAPD technique is performed at standard low annealing temperatures between 35 and 38°C though, band reproducibility can be problematic. By increasing the annealing temperature to over 46°C, the high annealing temperature RAPD (HAT-RAPD) technique has been shown to provide a highly reproducible degree of polymorphism[20].

In this paper, we use the HAT-RAPD technique to characterize the transmission of genomic DNA during the creation of new lychee hybrids. By characterizing gender-specific transmission rates as well as cultivar-specific HAT-RAPD bands, it is possible to follow the transmission of genomic information from parental strains to the hybrids and determine the ancestry for hybrids of unknown parentage. This improved understanding of the hybridization process in lychee could provide breeders with a new method to track variety relatedness, select potential new plants to grow to maturity, and to choose optimal parental combinations for trait propagation.

Materials and methods

Plant Material

Four commercially available lychee cultivars named Chacrapat, KimJeng, Hong Huey and O-Hia were chosen as parental strains to form the hybrids analyzed in this study. Hybrids were classified by the sex and variety of the parental cultivars. From each genetic cross, a total of three distinct offspring were then used for DNA analysis.

DNA Extraction

Isolation of total genomic DNA for the molecular marker analysis was carried out utilizing the cationic hexadecyl trimethyl ammonium bromide (CTAB) method of Weising et al.[21] (1991) with minor modifications to optimize for the lychee species. Initially, the tips of young leaf pieces (0.5 – 1.0 g) were washed twice in sterilized distilled water and ground to a fine powder with liquid nitrogen in a sterile mortar and pestle. The resulting powder (300 mg) was transferred to a 1.5 ml microcentrifuge tube, suspended in 700 ml preheated CTAB extraction buffer and then incubated at 60°C in a water bath for 60 minutes. Proteinase K (10 mg ml^{-1}) was then added and the tube was carefully inverted several times before being incubated at 37°C for 60 minutes. The tubes were then centrifuged at 12,000 rpm for 5 minutes and the supernatants were transferred to new tubes containing an equal volume of chloroform. The tubes were again inverted for 5 minutes and centrifuged at 12,000 rpm for 3 minutes. The resulting supernatants were transferred to new tubes and an equal volume of cold isopropanol was added, after which they were incubated at -20°C overnight. Finally the tubes were spun down at 12,000 rpm for 15 minutes and the supernatants removed. An additional wash with 500 ml of cold 70% ethanol was performed before the final spin down at 12,000 rpm for 5 minutes. The nucleic acid (DNA, RNA) pellets were dried at a room temperature and resuspended in 50 ml of TE buffer.

DNA Purification

To purify the DNA, 10 mg ml^{-1} of RNase A (Sigma) was added to the solution and incubated at 65°C for 15 minutes. Then, 300 ml of TE buffer and 300 ml of phenol was added. The tubes were inverted for 5 minutes and centrifuged at 10,000 rpm for 5 minutes. The supernatants were transferred to new tubes and an equal volume of chloroform:isoamyl alcohol (24:1) solution was added. The tubes were again inverted for 5 minutes, centrifuged at 10,000 rpm for 5 minutes, and the aqueous phase was transferred to a new tube. This procedure was repeated twice before the DNA was quantified using the UV absorbance at 260 nm of the DNA with a spectrophotometer (Beckman, DU-7500) and then by 0.7% agarose gel electrophoresis.

HAT-RAPD Analysis

For each lychee cultivar, 10 ng of the genomic DNA produced previously was amplified using the protocol of Anuntalabhochai et al.[20] (2000). The PCR reaction was performed in a total volume of 20 ml containing: 10xPCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl) (QIAGEN), 100 mM each dNTP (dATP, dTTP, dGTP, dCTP) (Promega), 1.5 mM $MgCl_2$ (QIAGEN), 0.3 mM 10-base primers (obtained from Operon Technologies Inc., Alameda, California), and 0.5 unit of *Taq* DNA polymerase (QIAGEN). The DNA was amplified in a Perkin Elmer thermal cycler (Gene Amp PCR system 2400) using the following cycling profile: an initial 2 min. at 95°C, then 95°C 30 sec., 46-55°C 30 sec., and 72°C 45 sec., for 35 cycles, and then a final cycle at 72°C 5 min. After the thermal cycling program was complete, the amplification reaction products were

stored at 4°C prior to the electrophoresis analysis.

DNA Fingerprinting

Ten arbitrary random primers (Table 1) were selected to detect the genetic variation within five hybrid combinations of lychee using the high-annealing temperature randomly amplified polymorphic DNA method (HAT-RAPD). Figures 1 and 2 show representative HAT-RAPD band patterns obtained using the following four hybrid combinations of lychee (female x male): Chacrapat x KimJeng, KimJeng x Chacrapat, KimJeng x Hong Huey, Hong Huey x KimJeng for the primers OPL-11 and OPC-09.

Results

For each of the 10 random primers and five hybrid pairs, HAT-RAPD analyses were run to characterize the conserved band markers, as shown in Figures 1 and 2. For the hybrid cross of Chacrapat x KimJeng, the number of bands conserved in the father, mother and hybrid are summarized in Table 2a. As can be seen from this table, there were 33 variety-specific bands conserved in Chacrapat not observed in KimJeng, 26 variety-specific bands conserved in KimJeng, and 80 bands shared by both varieties. Each random primer on average found approximately 15 bands, of which on average 2 – 3 were specific to a given variety.

Table 1. Primers used for the genetic analysis of lychee hybrids.

Primer used	Sequence (5' → 3')
OPL-11	TGGTGGACCA
OPC-09	CTCACCGTCC
OPH-13	GACGCCACAC
OPU-03	CTATGCCGAC
OPD-18	GAGAGCCAAC
OPG-03	GAGCCCTCCA
OPG-13	CTCTCCGCCA
OPD-20	ACCCGGTCAC
OPD-20	ACCCGGTCAC
OPW-19	CAAAGCGCTC

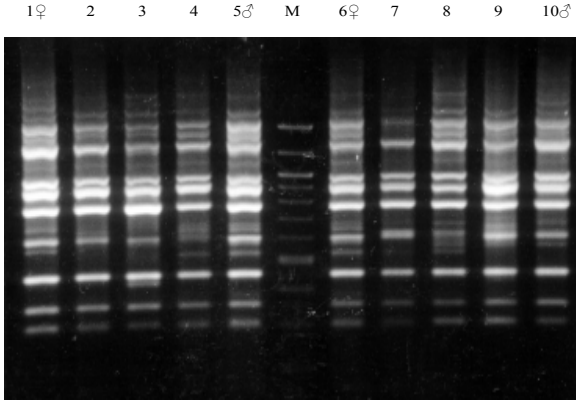

Fig 1. HAT-RAPD analysis of DNA from Lychee cultivars and hybrids using the OPL-11 primer. Lane 1, Chacrapat female; lanes 2-4, hybrids between the Chacrapat female and KimJeng male; lane 5, KimJeng male; lane 6, KimJeng female; lanes 7-9, hybrids between the KimJeng female and Chacrapat male; lane 10, Chacrapat male; lane M, molecular weight marker 100 bp ladder.

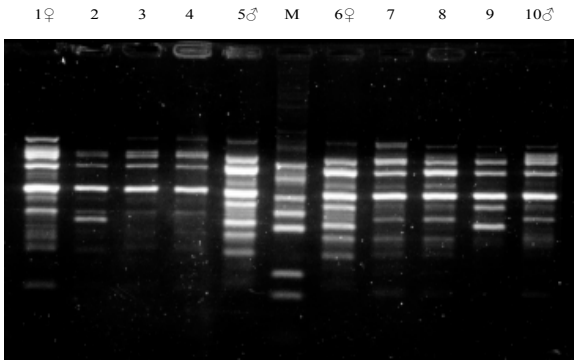

Fig 2. HAT-RAPD analysis of DNA from Lychee cultivars and hybrids using the OPC-09 primer. Lane 1, KimJeng female; lanes 2-4, hybrids; lane 5, HongHuey male; lane 6, HongHuey female; lanes 7-9, hybrids; lane 10, KimJeng male, and lane M, molecular weight marker 100 bp ladder.

Table 2a. Transmission of bands conserved in either parent and/or hybrid offspring for a single Chacrapat x KimJeng lychee cross.

Male ♂ Chacrapat	Female ♀ KimJeng	Offspring	Number of Bands
+	+	+	72
+	+	-	8
+	-	+	22
+	-	-	11
-	+	+	20
-	+	-	6
-	-	+	2

Gender Specific Band Transmission

To determine the rate of marker transmission, bands for each parent and hybrid were scored and the number of bands transmitted to each offspring was recorded as a percentage transmission rate, as shown in Table 2b. In all hybrid crosses, the transmission of bands was greatest when the band was initially found in both parents. For bands only found in the male or female parent, the transmission was reduced, but in all cases regardless of the cultivar variety, the female passed on significantly more bands than the male parent ($p = 0.015$). Since there was no (cultivar x gender) effect, the most accurate overall band transmission rate, which is presented in the last line of Table 2b,was determined by pooling all of the data. A low background of bands not occurring in either parental strain was also found,

Table 2b. Percentage of HAT-RAPD bands transmitted to hybrids.

Variety (M x F)	Percentage of bands transmitted to offspring			
	Both Parents	Female Only	Male Only	Neither
Chacrapat x KimJeng	90.0	76.9	66.6	1.4
KimJeng x Chacrapat	85.4	88.9	74.2	0.7
KimJeng x Hong Huey	84.0	73.3	51.4	1.4
Hong Huey x KimJeng	82.8	75.0	62.5	0.7
Chacrapat x O-Hia	87.5	75.8	64.0	0.0
Overall Transmission	85.9 ± 2.8	78.0 ± 6.2	63.8 ± 8.2	0.8 ± 0.6

as summarized in the last column of Table 2b.

Hybrid Detection

For an arbitrary cross of two lychee varieties where the specific genders of the individual parents are not known, the transmission rate by the male parent can be used as a lower bound on the number of bands transmitted. So, given a hybrid of unknown ancestry, if bands unique to only one parental variety are known, such bands can be used to characterize the ancestry of the hybrid. Unfortunately, in all cases, bands are not uniformly transmitted, so to provide an adequate chance of detecting cultivar specific bands, sufficient parental cultivar specific bands must initially be characterized to allow resolution of the true parental cultivar varieties. As discussed above, each random primer produced approximately 2 – 3 reproducible cultivar specific bands. For a 60% parental transmission rate, which is a conservative estimate from the measured transmission rates described above, Table 3 shows the probability of transmitting various numbers of bands, as predicted using the binomial distribution. If a specific plant is a hybrid of a given parental variety, as Table 3 shows, the probability of not passing a single band is 1% or less if at least 5 variety-specific HAT-RAPD bands were characterized. For more than 10 variety-specific bands, the chance of not transmitting at least 2 bands is less than 10^{-4}. Therefore given enough primers, the lychee parental varieties of interest can be ascertained with nearly statistical certainty. In addition, if 0 or 1 bands are found for a specific variety, then that variety can be excluded as one of the parental cultivars if more than 10 variety-specific bands have been characterized.

Table 3. Theoretical percentage of bands transmitted given a 60% hybrid transmission rate and the given number of variety specific bands. (0* denotes a less than 10^{-4} probability).

Variety Specific Bands	Number of transmitted bands			
	0	1	>1	>2
3	6.4	28.8	64.8	21.6
4	2.56	15.36	82.08	47.52
5	1.02	7.68	91.3	68.26
6	0.41	3.69	95.9	82.08
7	0.16	1.72	98.12	90.37
8	0.07	0.79	99.15	95.02
9	0.03	0.35	99.62	97.5
10	0.01	0.16	99.83	98.77
15	0*	0*	100	99.97
20	0*	0*	100	100

Discussion

Sources of polymorphisms in a HAT-RAPD assay may include base changes within a priming site sequence, deletions of a priming site, insertions that render priming sites too distant to support amplification, and deletions or insertions that change the size of a DNA fragment without preventing its amplification[19]. Differences in band markers in parents and offspring may be the result of DNA recombination, mutation, or random segregation of chromosomes in meiosis processing during hybridization[22,23]. In this study, 20.3 ± 3.9% of band markers from the parents were not found in any of the five hybrid combinations, and only 0.8 ± 0.6% of new band markers were found exclusively in the hybrids. The low number of bands not shared with parents in offspring of lychee is most probably due to segregation of heterozygous chromosomes during meiosis. Chromosomal crossing-over during meiosis may also result in the loss or rearrangement of priming sites and thus markers could be present in parents but not in offspring or vice-versa[24]. The phenomenon of non-Mendelian inheritance has also previously been detected due to the existence of competition in RAPD analysis in the pea[25,26]. Due to the heterozygous inheritance, it is not surprising to find only a portion of the bands from each parent in the lychee hybrid.

The 10 random primers used in this study yielded a wealth of polymorphic bands for the HAT-RAPD analysis and were able to provide approximately 2 – 3 variety-specific bands for each random primer. With an increasing the number of cultivar varieties examined, it is expected that the number of bands which are unique to only one cultivar variety would be reduced. However, it should be possible to characterize the entire range of 50-60 lychee cultivar varieties with just these 10 primers, since there are currently between 25 and

40 cultivar specific band markers characterized for each variety, which is more than is needed by a factor of three. Using this band marker data, hybrid detection and ancestry determination can be performed at a statistically highly significant level. As a method to distinguish and classify hybrids, the HAT-RAPD data described in this paper provides a significant resource for lychee breeders to detect unwanted hybrids before plants reach maturity, which could dramatically reduce the current uncertainties in lychee propagation.

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References

1. Batten DJ (1984) Lychee varieties. Agfact H6.2.7., Dept. Agric., Sydney, N.S.W., pp. 15.
2. Chapman KR (1984) Characteristics of common lychee varieties of China — in order of maturity. Maroochy Hortic. Res. Station, Nambour, Qld., Australia.
3. Subhadrabandhu S (1990) Lychee and Longan cultivation in Thailand. Rumthai Publication, Bangkok.
4. Crawford DJ (1990) Plant Molecular Systematics: Macromolecular Approaches. Wiley, New York, pp 249-52.
5. Agarwal K and Gupta PK (1983) Cytological studies in the genus *Medicago* L. *Cytologia* **48**, 781–93.
6. Dinelli G and Bonetti A (1992) Capillary electrophoresis in species and cultivar determination. *Seed Sci and Technol* **20**, 241–9.
7. Konarev V, Gavriljuk I, Gubareva N and Peneva T (1979) Seed proteins in genome analysis, cultivar identification and documentation of cereal genetic resources: a review. *Cereal Chem* **56**, 272-8.
8. Torres AM, Weeden NF and Martin A (1993) Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. *Theoret and Appl Genet* **85**, 937-45.
9. Naton B, Ecke M and Hampp R (1992) Production of fertile hybrids by electrofusion of vacuolated tobacco mesophyll protoplasts. *Plant Sci* **85**, 197-208.
10. Tingey SV and del Tufo JP (1993) Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiol* **101**, 349-52.
11. Wang ZY, Second G and Tanksley SD (1992) Polymorphism and phylogenetic relationships among species in the genus *Oryza* as determined by analysis of nuclear RFLPs. *Theoret and Appl Genet* **83**, 565-81.
12. Wangspa R, Cutler RW, Sitthiprom S, Chundet R, Dumampai N and Anuntalabhochai S (2005) DNA fingerprint database of some economically important Thai plants: *Litchi chinensis* Sonn., *Dimocarpus longan* Lour, and *Peuraria* spp. *Sci Asia* **31**, 145-9.
13. Clark AG and Lanigan CMS (1993) Prospects for estimating nucleotide divergence with RAPDs. *Molec biol Evol* **10:5**, 1096-111.
14. Cutler RW, Chundet R, Handa T and Anuntalabhochai S (2006) Development of sequence characterized DNA markers linked to a temperature dependence for flower induction in lychee (*Litchi chinensis* Sonn.) cultivars. *Scientia Horticult* **107:3**, 264-70.
15. Cutler RW, Sitthiphrom S, Marha J and Anuntalabhochai S (2006) Development of sequence characterized DNA markers linked to a temperature insensitivity for fruit production in longan (*Dimocarpus longan* Lour.) cultivars, *J. Agron and Crop Sci*, in press. doi: 10.1111/j.1439-037X.2006.00235.
16. Dunemann F (1994) Genetic relationships in *Malus* evaluated by RAPD fingerprinting of cultivars and wild species, Eucarpia Fruit Breeding Section Meeting, Aug 30-Sept 3, 1993, *Euphytica*. pp 321-2.
17. Rafalski JA and Tingey SV (1993) Genetic diagnostics in plant breeding: APDs, microsatellites and machines. *Trends Genet* **9**, 275-80.
18. Welsh J and McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 6531-5.
19. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* **18**, 6531-5.
20. Anuntalabhochai S, Chandet R, Chiangda J and Apavatjrut P (2000) Genetic Diversity within Lychee (*Litchi chinensis* Sonn.) based on RAPD analysis. *Acta Horticult* **575**, 253-9.
21. Weising K, Kaemmer D, Weigand F, Epplen JT, and Kahl G (1991) Oligonucleotide fingerprinting reveals various probe-dependent levels of informativeness in chickpea (*Cicer arietinum*). *Genome* **35**, 436-42.
22. Huchett BI and Botha FC (1995) Stability and potential use of RAPD markers in a sugarcane genealogy. *Euphytica* **86**, 117-25.
23. Darnell JE, Baltimore D and Lodish HF (1990) *Molecular Cell Biology*. Scientific American Books, Inc., pp 156-9.
24. Smith JF, Burke CC and Wagner WL (1996) Interspecific hybridization in natural populations of *Cyrtandra* (Gesneriaceae) on the Hawaiian Islands: evidence from RAPD markers. *Plant Systemat Evol* **200**, 61-77.
25. Lu J, Knox MR, Ambrose MJ, Brown JKM and Ellis THN (1996) Comparative analysis of genetic diversity in pea assessed by RFLP-and PCR-based methods. *Theoret Appl Genet* **93**, 1103-111.
26. Hallden C, Hansen M, Nilsson NO and Hjerdin A (1996) Competition as a source of errors in RAPD analysis. *Theoret Appl Genet* **93**, 1188-92.