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Identification of true to type and open pollinated progenies of polyembryonic *Mangifera indica cv.* Harumanis using microsatellite markers

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Abstract

The study was conducted on a polyembryonic mango, Harumanis which contains more than one embryos including one zygotic and single or more number of nucellar ones. In this paper, we used microsatellite markers to identify whether the seedlings of Harumanis were zygotic or nucellar. A total of 95 progenies of Harumanis were evaluated using 13 polymorphic microsatellite markers. The genetic profiling revealed that a total of 14 Harumanis progenies were zygotic or open pollinated seedling as their genetic profile was different with Harumanis at least at one loci. Nevertheless, 76 Harumanis progenies were true to type or nucellar seedlings as their genetic profile was similar to Harumanis. The remaining five progenies could not be identified with the call rates of the genotypic data. Identifying true to type or nucellar seedling is useful for nursery growers to determine the true to type Harumanis progenies from the seed. Meanwhile, the open pollinated seedling or zygotic seedlings are preferred by breeders as they are considered as a new variety which increases the mango genetic variability.

Key word: Mango, Harumanis nucellar, zygotic, microsatellite

Introduction

Harumanis is considered the "King of Mangoes," gaining popularity in Malaysia because of its deliciousness, and sweet and aromatic fragrance. Harumanis is polyemroyonic thus its seed contains more than one embryo, which may be both zygotic and nucellar or all nucellar embryos (Shukla *et al.*, 2004; Degani *et al.*, 1993). In mangoes, this trait is genetically controlled by a single dominant gene while the cultivar and environmental conditions influence the number of seedlings produced from a seed (Aron *et al.* 1998; Andrade- Rodríguez *et al.*, 2005). The nucellar tissue that covers the embryo sac forms the nucellar embryos and produces genetically identical seedlings to its parent plant (Aron *et al.*, 1998). These seedlings from nucellar embryos are true to type as their mother and are preferred by nursery growers to produce rootstocks because using them results in a more even orchard (Rao *et al.*, 2008). On the other hand, fertilization either by self or cross-pollination forms zygotic embryo. This type of embryo will produce new open-pollinated progenies imperative for the development of new mango varieties. Unfortunately, based on morphological criteria, it is difficult or not possible to identify whether the seedlings are derived from nucellar or zygotic embryos (Desai, 2004), making molecular markers imperative for identification purpose.

In general, the most vigorous seedling from each seed is used to produce rootstocks. Unfortunately, uneven orchards may occur because the nucellar seedling is not always the most vigorous (Rocha *et al.*, 2014) which lead to impractical identification of nucellar seedlings using morphological characteristics. To differentiate zygotic and nucellar embryos, researchers used genetic markers, including isoenzymes (Degani *et al.*, 1993), RAPD (Ochoa *et al.*, 2012), and ISSR (Rocha *et al.*, 2014). To our knowledge, no reports were published using microsatellite markers for identifying nucellar and zygotic types in mango seedlings. Hence, this is the first report that used microsatellite markers for nucellar and zygotic identification. Microsatellite markers are extensively used in plant genomic studies because of their highly polymorphic, abundant and multi-allelic nature, simple analytical procedure, and transferability across genotypes (Vieira *et al.*, 2016). Therefore, they are useful to increase the efficiency of classification and identification of mango accessions, giving necessary information for future biodiversity conservation and gene bank management.

Materials and methods

Genomic DNA Extraction: Young leaves from 95 Harumanis progenies and their respective mother plants were collected to extract the genomic DNA. All samples were obtained from the mango plot located at the Malaysian Agriculture Research and Development Institute (MARDI), Sintok, Malaysia (6° 28' 53''N, 100 ° 29' 00''E). We collected the leaves and stored them with silica gel in an airtight plastic bag for air drying. The leaves were punched into a 96-well plate containing stainless steel beads (2.3 mm diameter) to obtain small fragments and were immediately frozen at –80°C for a minimum of one night. The frozen tissue was ground using Tissue Lyser (Qiagen, Germany). We immediately added the extraction buffer (2 % CTAB, pH8 of 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 0.05 % β-mercaptoethanol) after the tissues were ground. We extracted the total genomic DNA following the protocol by Mace *et al.*

Table 1. List of SSR marker used in this study

No	SSR	SSR ID	Forward	Reverse	Ta
1	MiIIHR01	M1	GGATGCACAACAACAAGCAC	TCAGCAAGCAATCCCTTCTT	61.1
2	MiIIHR02	M2	CCCCAACATTTCATAAACACA	CCTCCTTACATGCCTCCTTG	61.1
3	MiIIHR04	M4	CGTTTTTGACCCTCTTGAGC	CCGCATACTTCCCTTCACAT	58.3
4	MiIIHR06	M6	CGCCGAGCCTATAACCTCTA	ATCATGCCCTAAACGACGAC	55.0
5	MiIIHR07	M7	GCCACTCAGCTAAATAGCCTCT	TGCAGTCGGTAAAGTGATGG	63.0
6	MiIIHR10	M10	CGATTCAAGACGGAAAGGAA	TTCAAGCACAGACGACCAAC	58.3
7	MiIIHR11	M11	CAGTGAAACCACCAGGTCAA	TGGCCAGCTGATACCTTCTT	61.1
8	MiIIHR15	M15	CTAACCATTCGGCATCCTCT	TCTGTGATAGAATGGCAAAAGAA	58.3
9	MiIIHR18	M18	TCTGACGTCACCTCCTTTCA	ATACTCGTGCCTCGTCCTGT	55.0
10	MiIIHR23	M23	TCTGACCCAACAAAGAACCA	TCCTCCTCGTCCTCATCATC	58.3
11	MiIIHR24	M24	GCTCAACGAACCCAACTGAT	TCCAGCATTCAATGAAGAAGTT	58.3
12	MiIIHR28	M28	GCGGTCGCAGACAAATTCTATAT	ACAACTCGAGATTGTCACATCTTT	58.3
13	MiIIHR32	M32	TGGTGGTGTTTGTTTGCAGT	ACCACCCGCAGTATTGAAAG	58.3

(2003). We measured the DNA quality and quantity on 0.8 % agarose gels and Fluoraskan Ascent (Thermo Fisher Scientific, United States), respectively.

SSR genotyping: A total of 13 SSR markers (Table 1) were selected from a previous study by Ravishankar *et al.* (2011). The PCR was conducted as described by Schuelke (2000) by ligating the primers (either forward or reverse) with a non-fluorescent labelled M13 sequence tail (TGTAAAACGACGGCCAGT) and an M13 adapter labelled with a fluorescent dye (FAM, PET, NED or VIC). We prepared the PCR reaction mixture to a final volume of 10 µL, containing 1x buffer (Invitrogen, United States), 10 µM each of forward and reverse primer, 5 µM fluorescence-labelled M13 adaptor, 2 µM of each dNTP (Invitrogen, United States), 0.1 µL of bovine serum albumin (BSA) as PCR enhancer and 1 U of Taq polymerase (Invitrogen, United States). We performed the amplification using GeneAmp® PCR System 9700 (Applied Biosystems, United States) as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 41 – 65 °C for 45 sec, and 72 °C for 45 sec, followed by a final extension at 72 °C for 5 min. After the amplification, the PCR product was multiplexed using up to four primers with a combination of four different fluorescent dyes. We mixed the products Hi-Di formamide and GeneScan 500 LIZ, which were used as the standard molecular weight ladder (Applied Biosystems, United States) before being resolved using ABI 3130xL Genetic Analyzer (Applied Biosystems, United States).

Statistical analysis: We used the GeneMapper Version 5 (Thermo Fisher Scientific, United States) to score the allele size. The generated electropherograms were scored and analysed as described by Arif *et al.* (2010). We considered plants exhibiting different size of alleles from the mother plant as zygotic. Furthermore, we recognized the amplified products as polymorphic based on the presence of different or multiple allelic sizes across the total samples. The percentage of allelic similarity of each progeny to that of the respective mother parent was calculated using Microsoft Excel.

Results and discussion

The screening analysis of 95 progenies using 13 polymorphic microsatellite markers revealed a total of 14 Harumanis progenies being the zygotic form of seedlings since their DNA profiles did not match with their mother plant with at least at one marker. Among these 14 seedlings, 10 seedling showed polymorphism with at least two markers whereas the remaining four seedlings showed polymorphism for more than three markers. These seedlings are the source of new genetic variation which will contribute to new variety development caused by open pollination. Meanwhile, a total 76 Harumanis progenies were found to be nucellar seedlings as their DNA profile generated by 13 polymorphic microsatellite markers was the same as Harumanis DNA profile. The details of the genotypic data are summarized in Table 2. This technique provides an accurate identification of true to type seedlings which will benefit local and commercial farmer to plant true to type seedlings. However, the remaining five progenies were difficult to be identified since the call rates of the genotypic data were too low probably failed during amplification process. In other study, as in cranberry plants (*Vaccinium macrocarpon*), polymorphisms in three primers of dominant RAPD markers could differentiate between nucellar and zygotic plants (Novy *et al.* 1994). Furthermore, when assessing the genetic diversity of 102 accessions of 'Ubá' mango tree, the genetic distance was untangled using three ISSR primers from the nine primers used, differentiating between two accessions. Thus, they were considered as nucellar seedlings. As in this study, only four seedlings (plant ID: 10, 65, 70 and 120) showed polymorphism for more than three markers whereas 10 seedlings showed polymorphism in less than three markers. The dendogram tree based on shared alleles genetic distance for Harumanis and their progenies was constructed (Fig. 1).

To date, application of molecular markers to identify true to type or true open pollinated hybrid in mango and citrus species were reported (De Oliveira *et al.*, 2002; Rocha *et al.*, 2014). Since the application of molecular markers allows early selections of seedling types, the time and space required to propagate seedlings are reduced (Chunwongse *et al.*, 2015). Since, Harumanis is a commercial mango variety in Malaysia, both types of seedling

Table 2. Genotypic call of the *Mangifera indica* cv Harumanis and their progenies using 13 microsatellite markers

ID	M1	M2	M4	M6	M7	M10	M11	M15	M18	M23	M24	M28	M32
H	A	A	A	A	A	A	A	A	A	A	A	A	A
6	A	A	A	A	A	A	A	A	A	A	0	A	A
7	A	A	A	A	0	A	A	A	A	A	A	A	A
8	A	A	A	A	A	A	A	A	A	A	A	A	A
10	B	B	B	B	B	A	A	B	A	B	B	B	B
11	A	A	A	A	A	A	A	A	A	A	A	A	A
12	A	A	A	A	A	A	A	A	A	A	A	A	A
13	A	A	A	B	A	A	A	A	A	A	A	A	A
15	A	A	A	A	A	A	A	A	A	A	A	A	A
16	A	A	A	A	A	A	A	A	A	A	A	A	A
17	A	A	A	A	A	A	A	A	A	A	A	A	A
19	A	A	A	A	A	A	A	A	A	A	A	A	A
20	A	A	0	A	A	A	A	A	A	A	A	A	A
21	A	A	A	A	A	A	A	A	A	A	A	A	A
22	A	A	A	B	A	A	A	A	A	A	A	A	A
23	A	A	A	A	A	A	A	A	A	A	B	A	A
24	A	A	A	A	A	A	A	A	A	A	A	A	A
25	A	A	A	A	A	A	A	A	A	A	A	A	A
26	A	A	A	A	A	A	A	A	A	A	A	A	A
27	A	A	A	A	A	A	A	A	A	A	A	A	A
28	A	A	A	A	A	A	A	A	A	A	A	A	A
29	A	A	A	A	A	A	A	A	A	A	A	A	A
30	A	A	A	A	A	A	A	A	A	A	A	A	A
31	A	A	A	A	A	A	A	A	A	A	A	A	A
32	A	A	A	A	A	A	A	A	A	A	A	A	A
33	A	A	A	A	A	A	A	A	A	A	A	A	A
35	A	A	A	A	A	A	A	A	A	A	A	A	A
36	A	A	A	A	A	A	A	A	A	A	A	A	A
37	A	A	A	A	A	A	A	A	A	A	A	A	A
38	A	A	A	A	A	A	A	A	A	A	A	A	A
39	A	A	A	A	A	A	A	A	A	A	A	A	A
40	A	A	A	A	A	A	A	A	A	A	B	A	A
41	A	A	A	A	A	A	A	A	A	A	A	A	A
42	A	A	A	A	A	A	A	A	A	A	A	A	A
43	A	A	A	A	A	A	A	A	A	A	A	A	A
44	A	A	A	A	A	A	A	A	A	A	A	A	A
45	A	A	A	A	A	A	A	A	A	A	A	A	A
46	A	A	A	A	A	A	A	A	A	A	A	A	A
47	A	A	A	A	A	A	A	A	A	A	A	A	A
48	A	A	A	A	A	A	A	A	A	A	A	A	A
49	A	A	A	A	A	A	A	A	A	A	A	A	A
50	A	A	A	A	A	A	A	A	A	A	A	A	A
52	A	A	A	A	A	A	A	A	A	A	A	A	A
53	A	A	A	A	A	A	A	A	A	A	A	A	A
54	A	A	A	A	A	A	A	A	A	A	A	A	A
55	A	A	A	A	A	A	A	A	A	A	A	A	A
56	A	A	A	A	A	A	A	A	A	A	A	A	A
57	A	A	A	A	A	A	A	A	A	A	A	A	A
59	A	A	A	A	A	A	A	A	A	A	A	A	A
60	A	A	A	A	A	A	A	A	A	A	A	A	A
62	A	A	A	A	A	A	A	A	A	A	A	A	A
63	A	A	A	A	A	A	A	A	A	A	A	A	A
64	A	A	A	A	A	A	A	A	A	A	A	A	A
65	B	B	B	B	A	A	A	B	B	B	B	B	0
66	A	A	A	A	0	A	A	A	A	A	A	A	A
67	A	A	A	A	A	A	A	A	A	A	A	A	A
68	A	A	A	A	A	A	A	A	A	A	A	A	A
69	A	A	A	A	A	A	A	A	A	A	A	A	A
70	B	B	B	B	B	A	A	0	A	B	B	A	B
71	A	A	A	B	A	A	A	A	A	A	A	A	A
73	A	A	A	A	A	A	A	A	A	A	A	A	A
75	A	A	A	A	A	A	A	A	A	A	A	A	A
77	A	A	A	A	A	A	A	A	A	A	A	A	A
78	A	A	A	A	A	A	A	A	A	A	A	A	A
82	A	A	A	A	A	A	A	A	A	A	A	A	A
84	A	A	A	A	A	A	0	A	A	A	A	A	A
89	A	0	A	A	A	A	A	A	A	A	A	A	A
94	A	A	A	A	0	A	A	A	A	A	B	0	A
95	A	A	A	A	A	A	A	A	A	A	A	A	A
96	A	A	A	A	A	A	A	A	A	A	A	A	A
98	A	A	A	A	A	A	A	A	A	A	A	A	A
99	A	A	A	A	A	A	A	A	A	A	A	A	A
100	A	A	A	A	A	A	A	A	A	A	A	A	A
102	A	A	A	A	A	A	A	A	A	A	A	A	A
101	A	A	A	B	A	B	A	A	A	A	0	A	0
104	A	A	A	A	A	A	A	A	0	A	B	A	A
106	A	A	A	A	A	A	A	A	A	A	A	A	A
107	A	A	A	A	A	A	A	A	A	A	A	A	A
108	A	A	A	A	A	A	A	A	A	A	A	A	A
109	A	A	A	A	A	A	A	A	A	A	A	A	A
110	A	A	A	A	A	A	A	A	A	A	A	A	A
113	A	A	A	A	A	A	A	A	A	A	A	A	A
112	A	A	A	A	A	A	A	A	A	A	A	A	A
114	A	A	A	A	A	A	A	A	A	A	B	0	A
115	A	A	A	A	A	A	A	A	A	A	A	A	A
116	A	A	A	A	A	A	A	A	A	A	B	A	A
117	A	A	A	A	A	A	A	A	A	A	A	A	A
118	A	A	A	A	A	A	A	A	A	A	A	A	A
120	B	B	B	B	B	A	A	0	A	B	B	B	B
121	A	A	A	A	A	A	A	A	A	A	A	A	A
122	A	A	A	A	A	A	A	A	A	A	A	A	A

H: Harumanis; A: Same allele call size as parent (Harumanis); B: Different allele call size from parent (Harumanis); 0: Missing data

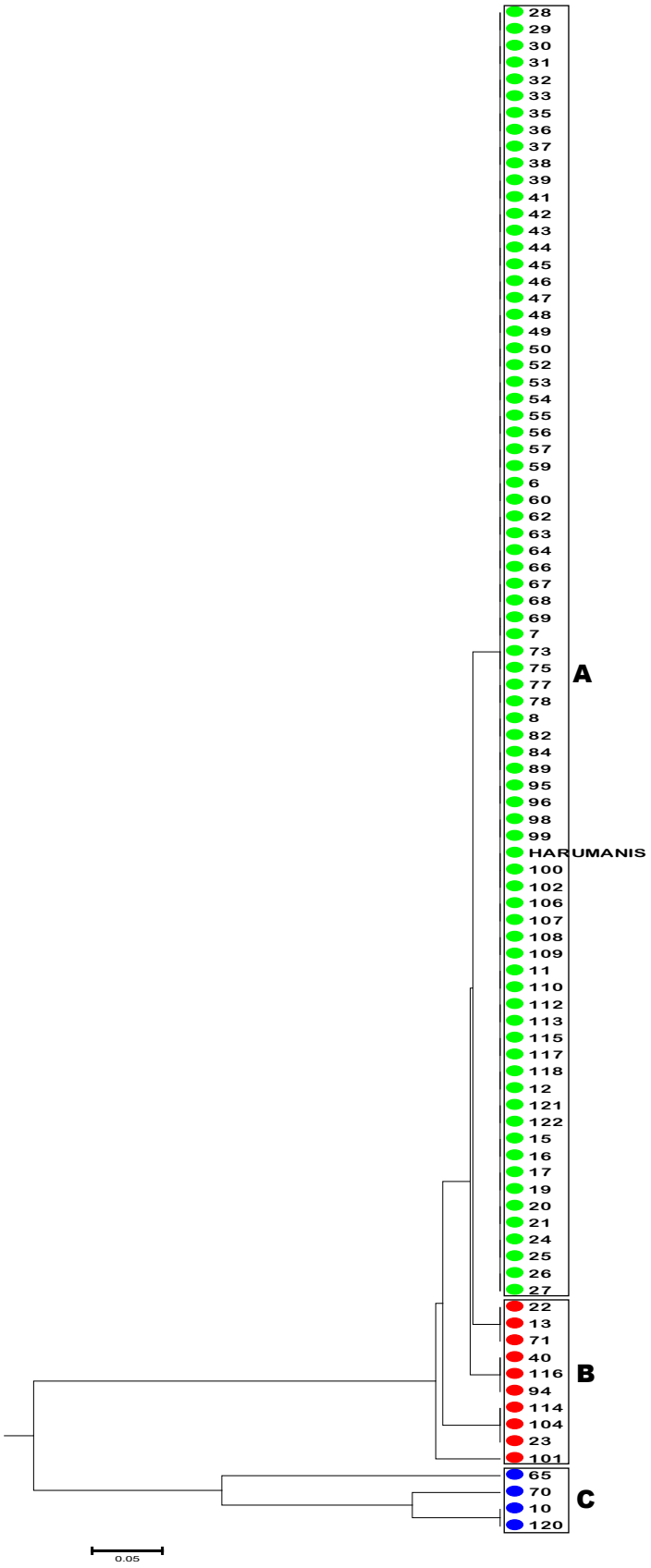

Fig. 1. UPGMA dendogram based on shared alleles genetic distance of *Mangifera indica* cv Harumanis and their progenies. A dots represents true to type seedlings; B dots represents progenies which differ from harumanis below than three loci; C dots represents progenies with different alleles with harumanis more than three loci.

are worthy. Zygotic seedling or true hybrid seedling is useful for breeders to develop new mango varieties in mango breeding program while nucellar seedling or true to type seedling is important for seedling producers. The polymorphism of the marker system can influence the efficiency in discriminating genotypes. As in this study, microsatellite markers were used to determine whether the embryos were zygotic or nucellar. Microsatellites have remained the most trusted markers for genotyping plants for the past 20 years and more owing to their useful characteristics, such as informative, highly polymorphic, multi-allelic, co-dominant, experimentally reproducible, and transferable across species (Vieira *et al.*, 2016). Moreover, the microsatellites are used in a wide range of genetic studies such as a diversity assessment measured in terms of genetic distance, cultivar identification through DNA fingerprinting, linkage and QTL analysis, and evolutionary studies (Kalia *et al.*, 2011).

In this study, microsatellite markers prove to be pivotal, capable of differentiating the embryo type of polyembryonic Harumanis seedlings. The identification of the seedling types is useful for breeders to develop new varieties and for seed producers to produce true to type seedlings.

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