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CHARACTERIZATION OF ZYGOTIC AND NUCELLAR SEEDLINGS IN POLYEMBRYONIC MANGO

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CHARACTERIZATION OF ZYGOTIC AND NUCELLAR SEEDLINGS IN POLYEMBRYONIC MANGO[1]

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ABSTRACT

Mangoes are classified as either monoembryonic or polyembryonic based on the numbers of embryos in the seed. Monoembryonic cultivars have a single zygotic embryo whereas polyembryonic cultivars have multiple embryos one of which can be zygotic and the rest are nucellar in origin. But it is difficult to identify and characterize the zygotic and nucellar seedling. Different morphological and biochemical markers have been used to distinguish nucellar from zygotic seedlings, but none is as efficient as molecular markers The Morphological characteristics was to evaluate the occurrence of polyembryony in the mango cultivars and to determine whether seedlings cultured in vitro are zygotic or nucelar. In one findings mango cultivars Manila and Ataulfo show polyembryony in more than 80% of their seeds, and the possibility of obtaining nucellar plants from them is high. Seed weight with the endocarp is an indicator of the number of embryos per seed. Another findings showed the three of eight rootstock mother trees of Turpentine were determined to be off-types. The single off-type seedling and the percentage of seedling detected by isozyme. The several findings also revealed the two different marker systems (dominant markers and SSR) were used which characterize the occurrence of zygotic or nucellar embryo in polyembryony mango cultivars. By using ISSR molecular markers to identify the genetic origin, zygotic or nucellar of seedlings from 'Ubá' mango polyembryonic seeds. The overall review concludes that the morphological, biochemical and molecular marker is very essential for characterization of zygotic and nucellar seedling.

Key Words: Mango, polyembryonic, Characterization, Seedling, Zygotic, Nucellar,

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CHAPTER 1

INTRODUCTION

The mango (*Mangifera indica* L) respected as one of the choicest natural products of the world, has a place to the family Anacardiaceae. It is considered to be the 'king of fruits', owing to its captivating enhance, delightful taste, powerful sweetness and alluring smell. In India mango are being related with horticulture and civilization from time immemorial (Begane *et al*., 2019).

Conventional mango cultivars from a specific topographical locale are hereditarily exceptionally comparable (Ravishankar *et al.,* 2000). Depending on the mode of generation of seeds mango can be classified into two bunches viz., monoembryonic and polyembryonic. Monoembryonic cultivars have a single zygotic embryo whereas polyembryonic cultivars have different embryos one of which can be zygotic (as a rule a powerless developing life) and the rest are nucellar in origin (Bally *et al*., 2009).

The nucellar embryos can be used for raising 'true-to-type' seedlings and the uniformity of seedlings is beneficial. Polyembryony is one of the impediments since the outcome of hybridization is the development of zygotic recombinants. The identification of resultant hybrid progenies of zygotic origin from that of nucellar embryony is difficult from a cross when one of the parents or both the parents used is a polyembryonic variety (Begane *et al*., 2019).

In Mexico the leading acknowledged cultivars within the household advertise are the polyembryonic ones, or yellow cultivars, such as Manila and Ataulfo (Ochoa *et al*., 2012). Mango plants are proliferated primarily by seed and grafting. Be that as it may, (Galvez-Lopez *et al.,* 2010). To guarantee the variety and maximum uniformity, it is essential to graft both monoembryonic and polyembryonic cultivars onto polyembryonic rootstock (Galán Saúco, 2009). In polyembryonic mango, there's one sexual fetus per seed and a few physical or nucellar ones, which share their whole hereditary structure with the mother plant Galán Saúco, 2009). Adventitious embryos are started straightforwardly from the maternal nucellar tissue, which encompass the developing life sac containing a creating zygotic embryo. (Aleza *et al.,* 2010).

The relationship of morphological characteristics of polyembryonic species with the zygotic beginning of the seedlings has been sought. (Ochoa *et al*., 2012). When mango is proliferated from polyembryonic seeds, ranchers permit them to sprout and deliver a few seedlings (counting sexually delivered plantlets). They select those with alluring characteristics and expect that this choice ensure the nucellar origin of the seedling (Galvez-Lopez *et al.,* 2010). Diverse

morphological and biochemical markers have been utilized to recognize nucellar from zygotic seedlings, but none is as productive as molecular markers (Rao *et al.*, 2008).

Isozymes have been used as biochemical markers to distinguish zygotic from nucellar seedlings in citrus (Moore and Castle, 1988). Isozymes are codominantly inherited, free from environmental effects, the analysis is nondestructive, and the assay is simple, which are all advantages over other methods (Schnell and Knight, 1992). Several enzyme systems have been used to develop a systematic characterization of a diverse array of mango cultivars. Degani *et al.*, (1990) found polymorphisms for six enzyme systems. Genetic inferences were made from gel isozyme patterns with a total of six loci and 17 allelomorphs identified. Use of a single enzyme system would result in off-type plants being missed if the zygotic plant had the same alleles as the maternal parent. The use of other enzyme systems in addition to GPI should enhance the ability to detect zygotics among seedling rootstock populations of polyembryonic cultivars (Schnell and Knight, 1992)

Mango has been the subject of many analysis using different molecular-marker types as RAPD (Souza *et al*., 2011), SCoT and ISSR (Luo *et al*., 2011), CAPS (Shudo *et al*., 2013), EST-SSR (Dillon *et al*., 2014), SSR and SNP (Sherman *et al*., 2015). The identification or differentiation of zygotic embryo among nucellar in polyembryonic seeds was examined by RAPD (Ochoa *et al*., 2012), and ISSR (Rocha *et al*., 2014). Dominant markers (RAPD, SCoT and SAP) and SSR marker to characterize the occurrence of zygotic or nucellar embryo in polyembryony mango cultivars Garifta Merah, Lalijiwo, Manalagi, Madu, Saigon Kuning, and Saigon Merah (Fatimah *et al*., 2016) were used to evaluate the utilization of two different marker systems.

Objectives:

The main purposes of this review paper are:

- To know the characteristics of polyembryonic mango cultivar
- To review biochemical and molecular identification of zygotic and nucellar seedlings in Polyembryonic mango.

CHAPTER 2

MATERIALS AND METHODS

This paper is absolutely a review paper. So, all the information's were collected from secondary sources with a view to prepare this paper. The title is selected with the consultations of my major professor. Various relevant books, journals which were available in the library of Bangabandhu Sheikh Mujibur Rahman Agricultural University and also in libraries of Bangladesh Agricultural University and Bangladesh Agricultural Research Institute were used for the preparation of this paper. For collecting recent information internet browsing were practiced. Good suggestions, valuable information and kind consideration from my honorable major professor and other teachers of the Department of Genetics and Plant Breeding, Bangabandhu Sheikh Mujibur Rahman Agricultural University help to enrich this paper. After collecting necessary information, it has been compiled and arranged chronologically for better understanding and clarification.

CHAPTER 3

REVIEW OF FINDINGS

3.1 Polyembryony mango

Mangoes are classified as either monoembryonic or polyembryonic based on the numbers of embryos within the seed. Monoembryonic cultivars have a single zygotic developing life though polyembryonic cultivars have different embryos one of which can be zygotic (ordinarily a powerless developing life) and the rest are nucellar in origin (Figure 1). The multiple nucellar embryos create adventitiously from nucellar tissues encompassing the fetus, and as a result, are hereditarily comparative to the tree bearing the seed (Juliano 1937). Polyembryonic seeds sprout as numerous isolated seedlings, most of which are nucellar in root and genuine to sort. In mango, the nucellar embryos are created from the nucellar tissue that encompassing the fetus sac, and the seedlings slid from these embryos are hereditarily comparable to the mother plant (Aron *et al.*, 1998). In any case, the zygotic fetus is determined from fertilization by self-pollination or by cross-pollination and the zygotic fetus is the objective in breeding programs for the choice of predominant genotypes and alluring characteristic (Rocha *et al.*, 2014).

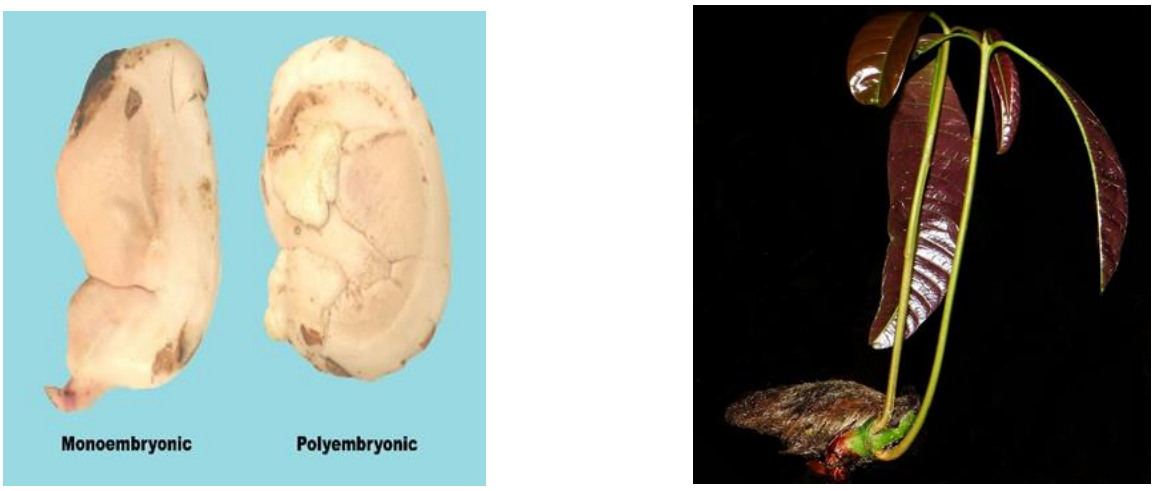

Figure 1. Embryos of monoembryonic and polyembryonic mango seeds and polyembryonic mango seedling .

(Source: Bally *et al*., 2009)

Utilizing polyembryonic cultivars for maternal guardians in a hybridization program is risky, as distinguishing proof and recuperation of the zygotic developing life is actually troublesome and as

it were conceivable by molecular screening methods (Degani *et al.*, 1992). For this reason most open and closed hybridisation breeding programs as it were utilize monoembryonic maternal guardians (Bally *et al.*, 2009).

3.2 Characterization of zygotic and nucellar seedling

Ploymbryonic seedling choice points to misuse the differences in polyembryonic populaces produced by characteristic change or out-crossed zygotic seedlings. It is well known that most polyembryonic seedlings are nucellar in root and genuine to the maternal parent type (Bally *et al.*, 2009). Mango breeders are challenged by the polyembryony problem, the phenomenon of multiple seedlings (one zygotic seedling and several nucellar seedlings) emerging from a single seed (Fatimah *et al*., 2016). This characteristic reduces the possiblity of finding out the true hybrid seedlings (Schnell and Knight, 1992).

Diverse morphological and biochemical markers have been utilized to recognize nucellar from zygotic seedlings, but none is as effective as molecular markers (Rao *et al.*, 2008). The random amplified polymorphic DNA (RAPD) are molecular markers characterized by their abundance in the genome; the utilization of two different marker systems, i.e. dominant markers (RAPD, SCoT and SAP) and SSR marker to characterize the occurrence of zygotic or nucellar embryo in polyembryony mango cultivars Garifta Merah, Lalijiwo, Manalagi, Madu, Saigon Kuning, and Saigon Merah (Fatimah *et al*., 2016).

3.2.1 Morphological characterization

'Manila' displayed 97% polyembryony and 'Ataulfo', 95%. Both cultivars had two to four embryos in more than 80% of their seeds (Table 1). According to Santos *et al.*, (2010), on the off chance that cultivars have polyembryony higher than 80%, the plausibility of getting nucellar plants increments, making it conceivable to have a uniform rootstock.

The traits of mango embryos have not been considered in previous studies, despite their importance for germination capacity (Andrade-Rodríguez *et al.*, 2004). In this study, embryos 3 and 4 weighed more and were longer, while embryos 1 and 2 were smaller (Table 2).

Table 1. 'Manila' and 'Ataulfo' seeds with different number of embryos

Cultivar	Number of embryos per seed							Polyembryony (%)	Embryos per seed
	1	2	3	4	5	6	7		
Manila	3	23	30	30	10	2	2	97	3.4a
Ataulfo	5	25	30	25	15	0	0	95	3.2a

Means followed by equal letters in the columns are not significantly different, by Kruskal-Wallis test, at 5% probability. Mean number.

(Source: Ochoa *et al*., 2012)

In 'Manila' and 'Ataulfo', a positive relationship was found between seed weight with endocarp (r=0.54) and number of embryos per seed (r=0.80). Within the previous cultivar, seeds with endocarp weighing between 13 and 18 g had bigger number of embryos, whereas, in 'Ataulfo', seeds weighing≥19 g had more embryos (Table 3). Deciding the relationship between seed characteristics and polyembryony is vital to anticipate which seed may contain more embryos, since bigger number of embryos within the seed increments their competitiveness and the plausibility that the zygotic developing life savages (Costa *et al.,* 2004). Contrasts in weight of up to 90% were found between the sets of cotyledons for all the assessed embryos (64 'Manila' embryos, and 54 'Ataulfo' embryos).

Table 2. Embryo traits according to their position in the seeds of polyembryonic mango 'Manila' and 'Ataulfo'

Embryo position	Fresh weight (mg)	Length (mm)	Width (mm)
1 (n = 40)	2087.30bc	26.40b	15.73a
2 (n = 36)	1256.50c	21.56b	14.59a
3 (n = 31)	4598.30a	38.80a	20.08a
4 (n = 11)	3658.20ab	36.30a	16.10a
CV (%)	102.00	47.21	74.33

Means followed by equal letters do not differ by Kruskal-Wallis test, at 5% probability. Embryo position 1 is the developing life another to the point of inclusion of the seed funiculus within the seed coat; the other fetus positions are organized counterclockwise and numbered agreeing to their position with regard to the funiculus.

(Source: Ochoa *et al.,* 2012)

In polyembryonic seeds, the contrast between cotyledons is likely due to compaction during embryos development, hindering their concurrent advancement (Sanchez-Damas *et al.,* 2006). In this way, evaluation of polyembryony ought to be particular for each class, species or cultivar. Out of the 60 primers assessed, 14 were chosen since they intensified the biggest number of strongly characterized bands (8 to 17 groups): OPA-1, OPA-2, OPA-4, OPA-11, OPA-18, OPB-6, OPB-7, OPB-10, OPB-12, OPB-18, OPC-14, OPC-19, SAP-1 and SAP-4. These groundworks increased 135 polymorphic groups for 'Manila', with 9.6 normal groups per preliminary, and 95 polymorphic groups for 'Ataulfo', with 6.8 normal groups per groundwork. These comes about moreover demonstrate hereditary contrasts between the two cultivars (Galvez-Lopez *et al.,* 2010).

Primer OPA-4 amplified the most elevated polymorphism for 'Manila' (94.4% of the groups, or 17 out of 18), as primer SAP-01 did for 'Ataulfo' (69.6%, 16 out of 23). No single primer by itself might distinguish all the zygotic seedlings, as Rajwana et al. (2008) had detailed. In any case, the set of primers OPA-02, OPA-04, OPA-11, OPB-07, OPB-10, OPB-12, OPC-14 and SAP-04 together recognized the zygotic embryos of both 'Manila' and 'Ataulfo' cultivars. With respect to the position of embryos within the seed (developing life area with regard to the funiculus), zygotic seedlings were found within the positions 1, 2, 3 and 5 in 'Manila', and positions 1, 2 and 3 in 'Ataulfo' (Table 4). In addition, zygotic seedlings were found primarily within the micropyle locale (positions 1 and 2) in 66.6% of 'Manila' polyembryonic seeds, and 57.1% of 'Ataulfo' polyembryonic seeds. Out comes about coincide with those of Cordeiro *et al.,* (2006).

Table 3. Average number of embryos in 'Manila' and 'Ataulfo' seeds according to their different weights with endocarp.

Seed weight (g)	Number of embryos per seed	
	'Manila'	'Ataulfo'
≤6.0	2.0	1.5
7.0–12.0	3.3	3.0
13.0–18.0	3.4	3.0
≥19.0	-	3.2

(Source: Ochoa *et al*., 2012)

Of the three monoembryonic seedlings from 'Manila' seeds (MeM-1, MeM-2 and MeM-3), as it were the seedling from the MeM-3 seed was recognized as zygotic by 10 primers (OPA-01, 02, 04, 11, 18, OPB-06, 07, 10, 12 and SAP-04). Seedlings MeM-1 and MeM-2 were distinguished as

nucellar, since they show the same banding design as the mother plant. In these seeds, it is conceivable that the zygotic seedling was not identified since it worsened, clearing out the nucellar developing life to create openly within the whole seed loculus (Batygina & Vinogradova, 2007).

Table 4. Localization of zygotic seedling in the polyembryonic mango 'Manila' and 'Ataulfo'.

Seed	Number of seedlings	Zygotic position
M 1	2	1, 2
M 2	5	5
M 3	3	0
M 4	3	1, 2, 3
M 5	2	0
M 6	5	2, 5
M 7	2	2
M 8	3	2
M 9	2	1, 2
MeM 1	1	0
MeM 2	1	0
MeM 3	1	1
A 1	3	1, 2, 3
A 2	3	1
A 3	4	0
A 4		2
A 5		0
A 6		0
A 7		2

Zygotic position 1 is the embryo next to the point of insertion of the seed funiculus in the seed coat. The other embryo positions were arranged counterclockwise and numbered according to their position with respect to the funiculus. M, 'Manila'; A, 'Ataulfo'; MeM, monoembryonic 'Manila' seeds.

(Source: Batygina & Vinogradova, 2007)

The degree of hereditary closeness among seedlings was decided by comparing the nonappearance or nearness of parts in assention with the mother plant banding design. The normal hereditary likeness among 'Manila' seedlings was 0.972 ± 0.034, and 0.977 ± 0.040 among 'Ataulfo' seedlings, with coefficients of variety of 0.034 and 0.041, separately. This demonstrates that the seedlings of both cultivars, notwithstanding of the root of the fetus, are profoundly comparative with a moo coefficient of variety (Rao et al., 2008). Considering these comes about and the moo likelihood of embryos within the positions 1 and 2 (for the most part zygotic) to sprout, 'Manila' and 'Ataulfo' cultivars would habitually create nucellar seedlings from seeds, as as of now noted by Rao et al., (2008).

3.2.2 Biochemical characterization

During the 1989 mango fruiting season (June-August), 25 seeds were collected from a single tree of each of the following rootstock cultivars: 13-1, Madoe, Sabre, and Golek. Turpentine rootstock seeds were collected from eight separate trees and bulked. It is important to know if a rootstock seedling is zygotic or nucellar in origin because of the possible effects on fruit yield and tree vigor (Schnell and Knight, 1992). Five isozyme systems (Degani *et al.*, 1990) were used to estimate the frequency of zygotics occurring within the most vigorous seedling populations. Stains were prepared as described by Soltis *et al.*, (1983) for the following enzymes: isocitrate dehydrogenase (EC l-1.1.42) (IDH), leucine aminopeptidase (EC 3.4.11.1) (LAP), glucose- 6-phosphate isomerase (EC 5.3.1.9) (GPI), PGM (EC 2.7.5.1), and triosephosphate isomerase (EC 5.3.1.1) (TPI).

Isozyme phenotypes were consistent with previous reports in mango (Degani *et al.*, 1990), with the exception of GPI (Figure 2). GPI generally has two loci that occur in most diploid plants. One locus is localized in the chloroplast, the other in the cytosol. In mango, two staining regions are apparent. The fast-migrating zone, labelled GPI-1, is monomorphic. The slow-migrating zone, labelled GPI-2, is polymorphic, and six or more phenotypes were observed: AA (one fast-migrating band); AB, the triple-banded phenotype that was not seen by Degani *et al.*, (1990), which may indicate another allele at this locus; AC, the phenotype analogous to Degani *et al.*, (1990) AB; and the CC phenotype. In addition to these patterns, several five-banded, four-banded, and two-banded patterns were seen in single seedlings from Golek and Madoe (Figure 2). These patterns are not easily explained using a single-locus model therefore, duplicated nuclear loci may occur (Schnell and Knight, 1992).

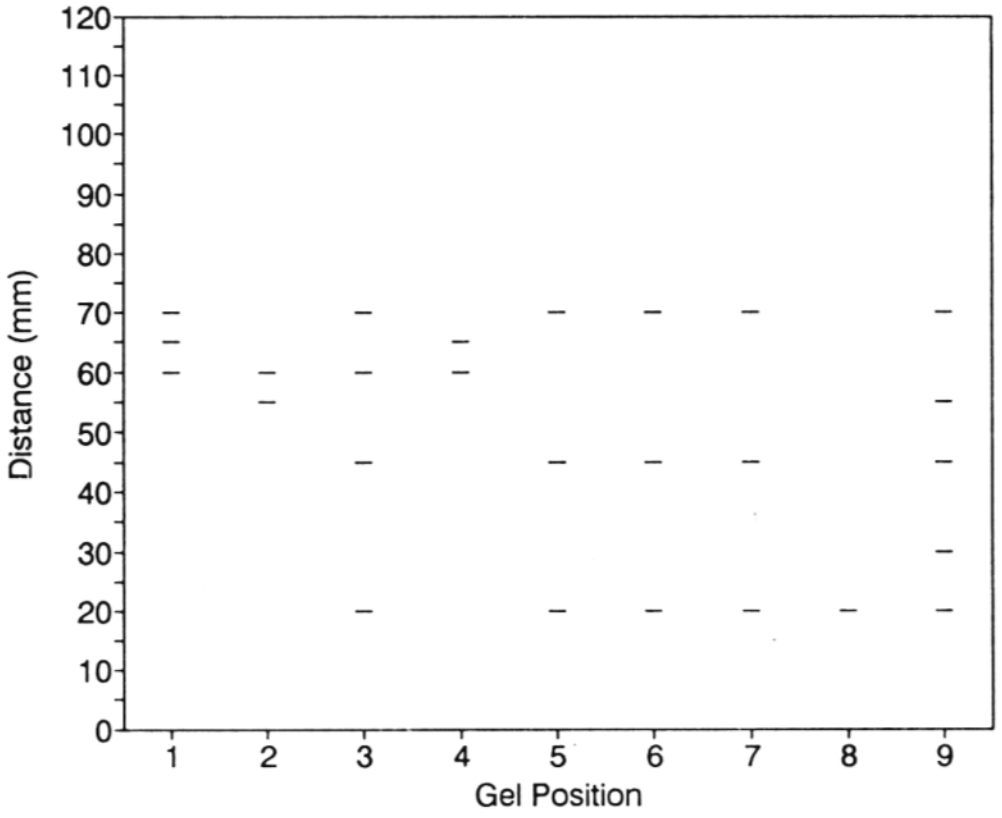

Figure 2. Isozyme banding patterns (photograph and corresponding diagram) for GPI phenotypes observed among rootstock cultivars and seedlings. Gel position: 1) Madoe, 2) Madoe RSP 1, 3) Madoe RSP 11, 4) Madoe RSP 18, 5) 13-1, 6) Turpentine, 7) Turpentine RSP 2, 8) Turpine RSP 9, 9) Golek RSP 13.

(Source: Degani *et al.*, 1990)

The seedlings from each rootstock were classified as maternal-type (nucellar) or offtype (zygotic) based on the isozyme phenotypes (Table 5). A contingency table was calculated and chi-square values estimated (Snedecor and Cochran, 1967) to determine whether observed differences in off-type frequencies were significant. With $x2 = 35.53$ (4 df and $P < 0.001$), significant differences existed between cultivars. The range of variation was from zero off-types in 13-1 to 16 of 25 in Golek (Table 5). The percentage of off-types reported for 13-l in Israel was from 10% to 15%, but we did not find off-types among the 25 seedlings of 13-1. This result may be attributed to the small sample size, to cross incompatibility with surrounding cultivars, or to the relative vigor of 13-1 nucellar embryos. The percentage of off-types in Sabre was also very low-4%. The percentages in

Turpentine and Madoe were high-24% and 36%, respectively. The number of heterozygous loci differed among the rootstock cultivars. Turpentine and 13-1 are heterozygous at four loci, Madoe is heterozygous at two, and Golek and Sabre heterozygous at one. The probability of detecting individuals from self-pollination was much greater in 13-1 and Turpentine seedlings because they had more heterozygous loci. No outcrossed or self-pollinated individuals were detected among seedlings of 13-1. Turpentine produced three plants that resulted from cross-pollination and three that could have resulted from self-pollination. All off-types of Madoe resulted from cross-pollination, while among off-types of Golek, 10 were from cross-pollination and six could have resulted from selfing. The single off-type seedling detected in Sabre was from crosspollination (Table 5). The percentage of seedlings resulting from self-pollination may be underestimated in Golek, Madoe, and Sabre because of the low number of heterozygous loci (Schnell and Knight, 1992).

Mango is polygamous, and some flowers are unisexual (staminate) while others are bisexual, both types being produced in the same panicle. Self- and cross-incompatibilities are known to exist in mango; however, the mechanism and degree of incompatibility are not understood. Turpentine and 13-1 were found to be self-incompatible in pollination studies (Gazit and Knight, 1989); therefore, it is unlikely that any of the seedlings from Turpentine or 13-l resulted from self-pollination. The fact that off-type seedlings of Madoe could not have resulted from selfing may indicate that this cultivar is also self-incompatible, but, to our knowledge, information on self- and cross-incompatibilities of Madoe, Golek, and Sabre does not exist (Schnell and Knight, 1992).

Table 5. Maternal parent, number of seedlings, percent offtypes, and isozyme phenotypes of parents and offtype seedlings for five polyembryonic mango cultivars

Maternal parent/seedling	Number of seedling	Percent offtypes	Isozyme phenotypes					
			GPI-2	ICDH	LAP	PGM-1	TPI	SOURCE
I3-I	25	0	AC	AC	AA	AC	AB	
SABRE	25	4	AA	CC	AA	AB	AA	
SABRE RSP 24						AC		e
TURPENTINE	25	24	AC	AC	AA	AC	AB	
TURP RSP 1			AA	AA	AB		BB	e
TURP RSP 7			AA				AA	d
TURP RSP 8			AA					d
TURP RSP 9			CC	CC			BB	d
TURP RSP 10			AA		AB			d
TURP RSP 11			AA		AB			e
MADOE	25	36	AB	CC	AA	CC	BB	
MADOE RSP 1			a				AB	e
MADOE RSP 2							AB	e
MADOE RSP 3							AB	e
MADOE RSP 4							AB	e
MADOE RSP 5							AB	e
MADOE RSP 10			AC	AC			AB	e
MADOE RSP 11			b	AC			AB	e
MADOE RSP 18			a	AC				e
MADOE RSP 25			AA	AC				e
GOLEK	25	64	AB	AC	AA	BB	AA	
GOLEK RSP 1			AB					d
GOLEK RSP 2			AC			AB		e
GOLEK RSP 4			AA					d
GOLEK RSP 5			AC	CC		AB		e
GOLEK RSP 6			AC					e
GOLEK RSP 7				AA				d
GOLEK RSP 8			c		AB	AB		e
GOLEK RSP 9						AB		e
GOLEK RSP 10						AB		e
GOLEK RSP 11						AB		e
GOLEK RSP 13			c	CC				e
GOLEK RSP 18			c					e
GOLEK RSP 21				CC				d
GOLEK RSP 23				AA				d
GOLEK RSP 24			c					e
GOLEK RSP 25				CC				e

(Source: Schnell and Knight, 1992)

3.2.3 Molecular characterization

3.2.3.1 Plant Materials

Fatimah *et al.*, 2016 were conducted an experiment which consisted of 6 polyembryonic mango cultivars (Table 6). Young and healthy leaf samples (maternal) and fruits (embryo) were collected from the germplasm collections of Cukur Gondang field station of Indonesian Tropical Fruit Research Institute, Pasuruan, and East Java. The endocarp and seed coat (testa with tegmen wrapping all embryos) were evacuated from each seed, and the number of embryos per seed was decided. The embryos were isolated and numbered concurring to their position with regard to the funiculus (Figure 3). The fetus another to the point of inclusion of seed funiculus within the seed coat was designated 'one'; the rest were arranged clockwise and after that numbered (Fatimah *et al.*, 2016). The funiculus was used as the reference, since in anatropous ovules, such as mango, it is next to the micropyle (Bachelier & Endress, 2009).

Table 6. List of mango accession used in this study

No.	ID	Accession	Origin
1	-	Garifta Merah	New Released Variety
2	53-54	Lali Jiwo-61	Kraksaan, Probolinggo
3	57-58	Madu-65	Pasuruan
4	61-62	Manalagi-69	Pasuruan
5	-	Saigon Merah	New Released Variety
6	-	Saigon Kuning	New Released Variety

(Source: Fatimah *et al*., 2016)

Figure 3. The seed and embryos performance of Saigon Kuning Variety. (A) The seed after the endocarp and seed coat were removed. (B) The embryos were separated and numbered according to their position with respect to the funiculus.

(Source: Fatimah *et al*., 2016)

3.2.3.2 Molecular Analysis

The PCR amplification was generated using Biorad Thermal Cycler PCR machine by following PCR conditions: (1) for SSR analysis used 16 primers (Schnell *et al*., 2006) (Table 7) (i) an initial denaturation step of 5 min at 94 0C, (ii) 30 cycles of 45 second at 94^0 C, 45 second at 55^0 C, 1 min at 72^0 C and (iii) a final extension step for 5 min at 72^0 C. (2) for dominant markers analysis used 16 primers (Ochoa *et al*., 2012) (Table 8), 44 cycles of 1 min at 94^0 C, 1 min at 35^0 C, 2 min at 72^0 C. Amplified products was separated by electrophoresis in 8% polyacrylamide gel (Dual Triple-Wide Mini-Vertical System, C.B.S. Scientific, CA, USA) for SSR analysis and Agarose gel for RAPD, SCoT and SAP followed by and observed by ethidium bromide and photographed under ultraviolet light using the gel documentation system (Fatimah *et al*., 2016).

Table 7. List of SSR primers used in this study

No	Primer	Forward	Reverse
1	AY942818	Ccacgaatatcaactgctgcc	tctgacactgctcttccacc
2	AY942820	aggtcttttatcttcggccc	aaacgaaaaagcagccca
3	AY942822	caacttggcaacatagac	atacaggaatccagcttc
4	AY942829	gaacgagaaatcgggaac	gcagccattgaatacagag
5	AY942831	tttaccaagctagggtca	cactcttaaactattcaacca
6	AJ938175	gctctttccttgacctt	tcaaaatcgtgtcatttc
7	AJ938179	tcggtcatttacacctct	ttattgagcttctttgtgtt
8	AJ635168	ttctaaggagttctaaaatgc	ctcaagtccaacatacaatac
9	AJ635170	Gacccaacaaatccaa	actgtgcaaaccaaaag
10	AJ635171	taaagataagattgggaagag	cgtaagaagagcaaaggt
11	AJ635172	tagggatatagctggagg	acgcagtagaacctgtg
12	AJ635175	tgcgtaaagctgttgacta	tcatctccctcagaaca
13	AJ635180	Cctcaatctcactcaaca	accccacaatcaaactac
14	AJ635182	gacttgcagtttcctttt	tcaagaaccccatttg
15	AJ635183	ccattctccatccaaa	tgcatagcagaaagaaga
16	AJ635187	atccccagtagctttgt	tgagagttggcagtgtt

(Source: Schnell *et al*., 2006)

Table 8. List of dominant markers used in this study

No.	Primer	Sequence
1	OPE6	Aagacccctc
2	OPF3	Cctgatcacc
3	OPF7	Ccgatatccc
4	OPG6	Gtgcctaacc
5	OPG13	Ctctccgcca
6	OPG19	Gtcagggcaa
7	OPH4	Ggaagtcgcc
8	SCoT61	caacaatggctaccaccg
9	SAP 1	atg cgaacc g
10	SAP 2	gac aca tcg g
11	SAP 3	tgg gac ctc c
12	SAP 4	gga gct acct
13	SAP 5	tat agg ccc t
14	SAP 6	cctact cca g
15	SAP 7	tgg gaa tcc c
16	SAP 8	gcc cct act a

(Source: Ochoa *et al.*, 2012)

3.2.3.3 SSR Analysis

Polymorphism Information Content (PIC) values, a reflection of allele diversity and frequency among the varieties, averaging 0.48 and ranging from a low of 0.07 (AJ938175) to a high of 0.84 (AJ635171) while AJ938175 was present the highest frequency allele up to 96% and AJ635171 showed the lowest frequency alleles 23%. Out of the 16 SSR primers evaluated, 9 primers amplified the largest number of allele and sharply defined band (4 to 9 bands): AJ635170, AJ635183, AJ635172, AJ938179, AY942831, AJ635175, AJ635168, AJ635187, and AJ635171 (Table 9) (Fatimah *et al*., 2016).

Table 9. Summary statistic of SSR primers.

Primer Name	Major allele frequency	Allele number	Band size (bp)	PIC
AJ938179	0.43	5	210-300	0.65
AY942822	0.5	2	200-280	0.38
AY942831	0.47	5	50-300	0.65
AY942829	0.48	3	370-450	0.49
AJ635171	0.23	9	50-280	0.84
AJ635170	0.6	4	160-250	0.51
AY942818	0.86	2	60-150	0.21
AJ635183	0.7	4	50-230	0.42
AJ635172	0.52	4	300-410	0.63
AJ635182	0.35	3	50-170	0.59
AJ938175	0.96	3	50-200	0.07
AJ635180	0.69	3	100-270	0.36
AJ635175	0.76	5	50-360	0.37
AY942820	0.82	2	50-230	0.25
AJ635168	0.59	5	50-200	0.56
AJ635187	0.32	6	70-320	0.67
Average	0.58	4	50-450	0.48

(Source: Fatimah *et al*., 2016)

3.2.3.4 Dominant Markers Analysis

The PIC values, averaging 0.44 and ranging from a low of 0.25 (SAP2) to a high of 0.50 (OPG13 and SAP5) while SCoT61 and SAP1 were present the highest frequency fragments up to 65% and 64% respectively and SAP2 showed the lowest frequency fragments 15% (Table 10) (Fatimah *et al*., 2016).

Table 10. Summary statistic of RAPD, SCoT and SAP primers.

Primer Name	The number of Fragment	Fragment frequency	Band size (bp)	PIC
OPE6	8	0,41	300-1800	0,48
OPF3	9	0,33	200-1300	0,44
OPF7	15	0,25	300-2000	0,38
OPG6	11	0,34	250-2000	0,45
OPG13	11	0,48	200-2000	0,50
OPG19	15	0,34	70-2000	0,45
OPH4	11	0,41	350-2000	0,48
SCoT61	4	0,65	400-1600	0,45
SAP 1	1	0,64	400	0,46
SAP 2	5	0,15	600-2000	0,25
SAP 3	10	0,39	200-2000	0,48
SAP 4	8	0,26	300-2000	0,38
SAP 5	3	0,51	400-600	0,50
SAP 6	5	0,39	100-1800	0,47
SAP 7	5	0,31	500-1500	0,43
SAP 8	5	0,28	200-1200	0,41
average	7,9	0,38	100-2000	0,44

(Source: Fatimah *et al*., 2016)

Furthermore, the PIC value can be determined based on the number and frequency of amplified fragments to measure the discriminatory power of a genetic marker system (Roldan *et al.*, 2000). In this study, the average PIC value of the evaluated SSR primers (Table 9) was higher than the PIC of dominant markers (Table 10) however in fact it was lower than determined PIC value for SSR system (maximum PIC value for co-dominat marker is 1.0) therefore it could not differentiate sharply while in dominant marker system, its PIC value of the evaluated dominant markers was high (Fatimah *et al*., 2016). The PIC value still confirmed the good discriminatory capacity of the primers as a maximum PIC values of 0.5 for dominant markers (De Riek *et al.*, 2001). That's why, the dominant markers systems could differentiate between zygotic and nucellar embryos clearly.

3.2.3.5 Identification of Embryo Type

By comparing the exhibiting of amplification patterns the type of embryo (zygotic or nucellar) was evaluated. Zygotic was identified as if different from the mother plant and nucellar as if they exhibited the same banding pattern as the mother plant. In SSR primers, the embryos of Garifta

Merah (E1, E2, E3, E4, and E5), Saigon Kuning (E2, E3, E6 and E8), Saigon Merah (E3), Lalijiwo (E2, E3, E4 and E5), and Madu (E1, E2, E5, E6, E7, E8, E9) were identified as zygotic while in dominant primers the embryos of Garifta Merah (E2, E3 and E5), Saigon Kuning (E1a, E2 and E6), Saigon Merah (E1 and E2), Lalijiwo (E2, and E3), Madu (E1,E2,E4,E5,E6,E7,E8,E9) and Manalagi (E1, E3, E5 and E6) were identified as zygotic. In the present study, the larger size of embryo (E1, E2, E3 and E4) and the small embryo (E5, E6, E7, E8, E9) both could be identified as zygotic embryo (Fatimah *et al*., 2016).

Table 11. Summary of zygotic embryo based on SSR and dominant markers (RAPD, SCoT and SAP) analysis

Mango Cultivar	Embryo per seed	SSR Analysis	
		Materna/ Nucellar	Zygotic
Garifta Merah	E1-E5	-	E1,E2, E3,E4,E5
Saigon Kuning	E1-E8	E1a, E1b, E4,E5,E7	E2,E3,E6,E8
Saigon Merah	E1-E5	E1, E2, E4, E5	E3
Lalijiwo	E1-E5	E1	E2, E3, E4, E5
Madu	E1-E9	E3, E4	E1,E2, E5, E6,E7,E8,E9
Mango Cultivar	Embryo per seed	Dominant markers Analysis	
		Materna/ Nucellar	Zygotic
Garifta Merah	E1-E5	E1, E4	E2, E3, E5
Saigon Kuning	E1-E8	E1b, E3, E4, E5,E7,E8	E1a, E2, E6
Saigon Merah	E1-E5	E3,E4,E5	E1, E2
Lalijiwo	E1-E5	E1, E4,E5	E2, E3
Madu	E1-E9	E3	E1,E2,E4,E5,E6, E7,E8,E9
Manalagi	E1-E6	E2,E4	E1,E3, E5,E6

(Source: Fatimah *et al*., 2016)

The percentage of zygotic embryos from six evaluated polyembryonic Indonesian mangoes cultivars derived from SSR marker analysis revealed 64% zygotic while from the evaluated dominant markers was 47% (Table 11). Schnell *et al.* (1994) reported that up to 66% of zygotic off types in different varieties of polyembryonic mango. The lowest number of zygotic embryos (higher number of nucellar/maternal embryos) was Saigon Kuning and Saigon Merah (~30%). However no single SSR or dominant primers by itself could identify all the zygotic embryos.

Therefore the set of those nine primers together could detect the zygotic/nucellar embryos of those cultivars (Fatimah *et al*., 2016).

3.2.3.6 Identification of nucellar and zygotic seedling

(Rocha *et al*., 2014) to identify the genetic origin, zygotic or nucellar of seedlings from 'Ubá' mango polyembryonic seeds by using ISSR molecular markers. The five seeds of accession 102, which originated 21 seedlings, zygotic seedlings were found in seeds 2, 4 and 5. Zygotic seedling were found in three seeds (2, 4 and 5), that is, 60% of the five seeds analyzed in accession 112 which yielded a total of 26 seedlings. The five evaluated seeds of accession 138 produced a total of 19 seedlings. The presence of zygotic seedlings was found in 18 of the 30 evaluated seeds (60% of the evaluated seeds) and the zygotic seedling was the strongest in six seeds (Table 12). These results indicated that the most vigorous seedling is not always genetically equal to the mother plant because the zygotic seedling was the most vigorous in 20% of the evaluated seeds. The same was found by Andrade-Rodriguez *et al*. (2004) using RAPD markers in *Citrus volkameriana*.

Table 12. Seedlings evaluated from 30 seeds (Sem) of five accessions (Aces) of Uba mango trees with their respective polyphormic (PP) and monophormic (PM) primers, used to identify zygotic and nucellar seedlings by ISSR markers

Aces	Sem	Seedling	Seedling	Seedling
			1, 2, 3, 4, 5, 6, 7	1, 2, 3, 4, 5, 6, 7
102	1	**1**, 2	0, 0	7, 7
	2	**1**, 2, 3, 4, 5, 6, 7	0, 0, **6**, 0, 0, 2, 2	7, 7, 1, 7, 7, 5, 5
	3	**1**, 2, 3, 4	0, 0, 0, 0	7, 7, 7, 7
	4	**1**, 2, 3, 4	**3**, 0, 2, 1	4, 7, 4, 6
	5	**1**, 2, 3, 4	0, 0, 0, **3**	7, 6, 7, 4
112	1	1, **2**, 3, 4, 5, 6	0, 0, 0, 1, 1, 2	7, 7, 7, 6, 6, 5
	2	1, **2**, 3, 4, 5, 6	**3**, **3**, 0, 0, 0, 0	3, 1, 7, 7, 7, 7
	3	**1**, 2, 3, 4	1, 0, 1, 1	6, 6, 6, 6
	4	**1**, 2, 3, 4, 5	0, **3**, 0, 0, 1	7, 4, 7, 7, 6
	5	**1**, 2, 3, 4, 5	**3**, 1, 0, 0, 0	4, 6, 7, 7, 7
138	1	1, **2**, 3	0, 0, 2	7, 7, 5
	2	**1**, 2, 3, 4, 5	0, 1, 0, 1, 1	7, 6, 7, 6, 6
	3	1, **2**, 3, 4	0, 0, 0, **4**	7, 7, 7, 2
	4	**1**, 2, 3, 4	0, 1, 1, **4**	7, 6, 6, 2
	5	**1**, 2, 3	0, 0, 2	7, 7, 4

Number underlined on the seedling column are the most vigorous seedling and on the polyphormic primers columns they correspond to the seedlings from zygotic embryos.

(Source: Rocha *et al*., 2014)

3.3 Significance of Polyembryony

Polyembryoni or nucellar embryonic plays an important role in Horticulture, Cytogenetics and Plant Breeding (Michael 2006).

- Nucellar embryonic helps in producing genetically uniform seedlings of the parental type for better clones of scion and rootstock.
- Polyembryoni helps in the large scale propagation of desired genotype.
- The nucellar seedlings show a restoration of the vigor lost after repeat vegetative propagation.

- The nucellar embryos are free from diseases as in-vitro nucellar embryonic is the only practical approachto raise virus free clones.
- Haploids can be used for cytogenetic studies.
- Homozygous diploids can be raised from haploids by colchicine treatment.

3.4 Limitation of Polyembryony

- Hybridization in polyembryonic mango. This phenomenon reduces the chance of recovering true hybrid seedlings.
- Different morphological and biochemical markers have been used to distinguish nucellar from zygotic seedlings, but none is as efficient as molecular markers. But molecular markers are costly.
- The main problem with this method and the reason it has not been traditionally favoured by plant breeders, is the ability to identify the zygotic embryos in the seed.

CHAPTER 4

CONCLUSION

- Different morphological and biochemical markers have been used to distinguish nucellar from zygotic seedlings, but none is as efficient as molecular markers.
- Mango cultivars Manila and Ataulfo show polyembryony in more than 80% of their seeds, and the possibility of obtaining nucellar plants from them is high. Seed weight with the endocarp is an indicator of the number of embryos per seed. Zygotic seedlings are not always produced by small embryos located at the micropylar end of the seed.
- The single off-type seedling and the percentage of seedling detected by isozyme. The zygotic seedlings identified is need to be evaluated as rootstocks to see if they affect productivity of the scion cultivar.
- The percentage of zygotic embryos from six evaluated polyembryonic Indonesian mangoes cultivars derived from SSR marker analysis revealed 64% zygotic while in dominant markers was 47%. By using ISSR molecular markers to identify the genetic origin, zygotic or nucellar of seedlings from 'Ubá' mango polyembryonic seeds.

CHAPTER 5

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