Study of polyembryony and development of molecular markers for identification of zygotic and nucellar seedlings in Khasi mandarin (*Citrus reticulata* Blanco)

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Abstract—The objective of this work was to evaluate the occurrence of polyembryonic seedlings and other morphological parameters in Khasi mandarin during three harvest years and to identify zygotic (sexual) seedlings from nucellar (asexual) ones grown under in-vitro conditions using molecular markers. Embryos from 27 polyembryonic and 7 monoembryonic seeds of Khasi mandarin were grown in-vitro. DNA from seedlings and mother parent was analyzed using 16 ISSR and 5 RAPD primers, of which 4 ISSR and a set of 3 RAPD primers were effective to identify zygotic or nucellar origin of the seedlings. In-vitro culture enables maximum embryos of each seed to grow, favouring the origin of seedlings to be identified as zygotic. Among 69 tested individuals, 37 zygotic and 32 nucellar seedlings were recognized. In polyembryonic and monoembryonic seeds, 59.6% and 42.8% of the seedlings, respectively, have the sexual origin. Morphological characteristics of seeds and the seedlings generated varied significantly and were not correlated with polyembryony except for the clutch size and the number of branches. Polyembryonic seeds in the cultivar are high, ranging from 50.0%, 55.5% to 83.3% over three harvest years with more clutch size and the possibility of obtaining zygotic plants from them is high. In polyembryonic seeds not all zygotic seedlings were produced by small embryos located at the micropyle. Identification of zygotic seedlings by ISSR and RAPD markers in Khasi mandarin cultivar is efficient and reliable at an early developmental stage.
Keywords— Khasi mandarin, molecular markers, polymorphism, zygotic seedlings.

I. INTRODUCTION

Khasi mandarin (*Citrus reticulata* Blanco) is a commercially popular fruit crop of the Eastern Himalayas exhibiting the common reproductive trait polyembryony (Nakano et al., 2012). Polyembryony has serious significance in citrus breeding since nurserymen use the genetically uniform healthy offspring to revive the old clones that have lost their vigour through regular vegetative propagation (Mondal and Saha, 2014). When citrus is propagated from polyembryonic seeds, the farmers allow them to grow and produce several seedlings (including hybrid and genetically uniform ones) for a long juvenile period of 6-7 years and they normally rogue off the hybrid ones, choosing those with desirable traits and presume that this selection guarantee the origin of the seedling (Mondal et al., 2014). However, this technique is not reliable due to some unusual developments which take place during embryo maturity resulting in the formation of good proportion of twin and triplets in the population (Mondal et al., 2015). In an open-pollinated population, the morphological identification of zygotic embryos becomes even more difficult. Moreover, the orchards are suffering from decline syndrome due to genetic erosion of proper planting material. To assure the variety and good vigour, it is indispensable to identify zygotic seedlings from nucellar ones as zygotic ones are vigorous and can compete with nucellar ones (Andrade-Rodriguez et al., 2005) for establishment of new *Citrus* orchards. Thus, it is imperative to develop efficient methods for differentiating zygotic embryos from nucellar ones at an early stage, for breeding purpose in polyembryonic cultivars. Several PCR based systems are available for genomic DNA analysis, amongst which ISSR, as well as RAPD markers are simple, reproducible, and user-friendly for farmer populations (Rao et al., 2008). These markers enable early selection of the progeny and this quality is particularly useful when a mixed hybrid population has to be rapidly analyzed and differentiated (Goodwin et al., 1997). Though these markers have been proven to identify origin of seedlings in different citrus crosses such as Mandarin (*Citrus reticulata*) and Pummelo (*Citrus maxima*), Yashar [(*Citrus changsha*) × (*Citrus paradisi* × *Citrus reticulata*)], Changsha (*Citrus Changsha)* and Ponkan (*Citrus reticulata*) (Rao et al.,

2008; Golein et al., 2011), but no investigation have been reported using molecular approaches for identification of origin of seedlings in Khasi mandarin cultivar mainly from an open-population system and this finding may considerably contribute to orchard management programs for this cultivar in the Indian subcontinent. The objective of this work was to evaluate the occurrence of polyembryonic seedlings as well as other morphological parameters collected during three harvest years in Khasi mandarin and the hypothesis tested was to see if these harvest years influence on the morphological parameters in the cultivar. The study also aimed to identify zygotic (sexual) seedlings from nucellar (asexual) ones in Khasi mandarin grown under *in-vitro* conditions.

II.   MATERIALS AND METHODS

2.1   Plant material

The seeds were collected from an open-pollinated Khasi mandarin (*Citrus reticulata*) trees (around fifteen years old) growing in a citrus farm located in Boko (25.97°N and 91.23°E), Assam, India. Matured and healthy fruits of Khasi mandarin were randomly harvested from six clonal mother trees located in close proximity to each other were collected during three harvest years of November in 2013, 2014 and 2015. Five fruits per plant were collected, amounting to a total of 30 fruits at each time of harvest. Five seeds per fruit were randomly selected to complete a set of 150 seeds and three replications of 150 seeds were used for each harvest year.

2.2   Study of polyembryony and morphological characteristics

To study polyembryony and morphological characteristics, the seeds were surface sterilized as described by Yun et al. (2007). The endocarp and seed coat (testa with tegmen covering all embryos) from each seed were removed and the size (length, mm) of the embryo (measured from the tip of the radicle to the opposite tip of the largest cotyledon) were recorded. The percentage of polyembryonic seeds, the number of embryos per polyembryonic seed and the average number of embryos or clutch size (Kishore et al., 2012) in polyembryonic seeds were recorded. The calculation of the average number of polymebryonic seeds were done by dividing the number of polyembryonic seeds by the total number of seeds and as a whole was calculated by dividing the total number of embryos by total number of seeds (both polyembryonic and monoembryonic) at each harvest.

For polyembryony studies, embryos from 27 polyembryonic and 7 monoembryonic seeds was grown *in-vitro* following Andrade-Rodriguez et al. (2005). *In-vitro* germination data were observed and recorded for three harvest years. The emergence of different seedlings

was noted and the number of leaves/seedling, number of branches/seedling, and shoot length/seedling were recorded. Representative embryos from the open pollinated seed population were evaluated to find suitable markers for identifying the zygotic seedlings.

2.3   Plant sample for polymorphism study

Fully expanded leaves from the six mother plants (from which the fruits were collected initially), were collected in plastic bags and kept at -80 °C until DNA extraction. The leaves from *in-vitro* germinated seedlings were also collected and placed in 2 ml eppendorf tubes, labelled and placed on ice until DNA extraction. Six mother plants, 62 seedlings from polyembryonic seeds and 7 seedlings from monoembryonic seeds were used for identification of zygotic seedlings using ISSR and RAPD markers.

2.4   DNA extraction

Total genomic DNA was extracted according to the method described by Doyle and Doyle (1990). DNA concentration and purity were quantified using a Nanodrop 1000 spectrophotometer (Invitrogen, Waltham, Massachusetts, USA) at 260/280 nm absorbance.

2.5   PCR amplification

PCR amplification was carried out in a Veriti thermal cycler (Applied Biosystems, USA) in a final volume of 25 µl reactions containing 25 ng of template DNA, 0.1 mM total dNTPs, 0.3 µM primer, 2.5 µl of 1X PCR buffer with 15 mM of magnesium chloride and 0.5 unit of Taq DNA polymerase (Bangalore genei, Bangalore, India). A total of 50 ISSR primers developed by University of British Columbia, Canada and 10 RAPD primers were custom synthesized from Eurofins Genomics (Bangalore, India). Initially two representative samples each from mothers and progenies were analyzed by PCR amplification. About 10 µl of PCR-amplified product (with 2 µl of 6X loading buffer) was analyzed on a 2% agarose gel in 1X TAE buffer stained with 10 mg/ml of ethidium bromide for visualization of bands for 3 h at 80 V and examined under UV transilluminator using the UVitech gel documentation system (Bangalore genei, Bangalore, India). Molecular weights of the PCR products were estimated by comparing them with 100 bp (100 µg/ml) and *ØX174* DNA /*HaeIII* digest (500 µg/ml) DNA ladders (Bangalore genei, Bangalore, India).

2.6   Data analysis

Mean and standard error values were calculated to reveal statistical significance for the morphological parameters evaluated per harvest year. The data (means of replications) was carried out in triplicates and statistical comparison of data was performed by ANOVA. The Pearson correlations analysis was done using R software 3.2V. A probability value of $p \leq 0.05$ was adopted as the criteria for significant differences. Duncan's multiple range (DMR) test was used for comparison between the

means of variables which was generated by the SAS program (version SAS 9.3, SAS Institute Inc., Cary, NC, USA). Scoring for polymorphism of the markers was carried out using a band-based method where bands were unambiguously scored as 1 and 0 for present and absent alleles respectively (Bonin et al., 2007). Both polymorphic and monomorphic loci were analyzed. Bands that resolved poorly on the gel were treated as missing ones.

III. RESULTS AND DISCUSSION
3.1 Polyembryony
In Khasi mandarin, polyembryony varied from 50.0% to 83.3% among three years and was recorded to be the highest in the seeds harvested in November 2013 while those harvested in November 2015 represented the lowest percentage of polyembryony. The percentage of germination was highest in the harvest year November 2015 (Table1). Factors influencing the variation in polyembryony found in our study and those reported by Andrade-Rodriguez et al., 2005 in *Citrus reshni* and Kishore et al., 2012 in *Citrus jambhiri* (90.1% and 91.4%) as well as germination traits could be attributed to genetic conditions (Kepiro and Roose, 2007), complex interrelation between genotype and surroundings such as the type of pollinators, quantity of viable pollens, fertilization, air temperature, environmental and soil humidity, plant nutrition, and wind speed (Andrade-Rodriguez et al., 2005; Mondal et al., 2014). Polyembryony varies depending on the ecological region and cultivar suggesting that it should be specific for each variety with respect to the region. The study indicated that polyembryony does not seem to be a limiting factor for germination capacity. Scalon et al. (2003) also reported that polyembryony was not a limiting factor for seed germination and plant emergence in *B. Glabra*. The number of embryos in polyembryonic seed ranged from 2 to 14 (Table 1), characterized by variation in the partitioning of embryos followed by both synchronous and asynchronous development of seedlings (Fig. 1a and 1b).

The average number of embryos (clutch size) per polyembryonic seed ranged from 2.27 ± 0.03 to 2.70 ± 0.10 and per total seed ranged from 1.13 ± 0.19 to 2.30 ± 0.12. One of the causes in the differences of clutch size during harvest years might be due to the presence of dominant trait for polyembryony, movement of auxins and ploidy level which plays prominent roles in the development of extra numerous embryo (Jaskani et al., 2005). Improper endosperm growth due to lack of nutrients and growth factors from the maternal tissue to the embryo might also lead to the early breakdown of nucellar embryos (Kishore et al., 2012). Our results

corroborate the findings of Kishore et al. (2012), where the number of embryos per seed reported in citrus (including mandarin, sweet orange, rough lemon, and lime) ranged from 2 to 14 while, it was different in terms of number of embryos per polyembryonic and total seed as reported by them (3.48 and 3.26). This significant disparity in the occurrence of clutch size evidently points out the impact of location on occurrence of numerous embryos. This suggests that the difference might be due to stress-induced changes in the environment leading to change in the genetic development of cells or their hormonal behavior, thereby affecting embryo development and surrounding seed organization (Batygina and Vinogradova, 2007). The number of leaves and shoot length were maximum in November 2015 and were minimum in November 2013, respectively, while the number of branches was the highest in November 2013 and the lowest in 2015 (Table 1). The variation in the emergence of the morphological traits suggests that polyembryony was only correlated with the number of branches indicating that it can be treated as a reliable indicator of the occurrence of polyembryony in the seeds. The variation could be due to the fact that the embryos of each seed were grown *in-vitro* using conical flasks during the harvest years for each seed which enabled development of a seedling from every embryo as compared to other studies which utilized seedlings grown in greenhouse, pots, etc (Golein et al., 2011; Yun et al., 2011). Moreover, during seed formation, embryos adapt to different dynamics especially when adventitious polyembryony is considered (Batygina and Vinogradova, 2007). One way Anova analysis was carried out at 0.05% significance where the probability Pr value 0.97 is greater than 0.05 (Table 2) which resulted in the rejection of null hypothesis suggesting that there is no significant variation in time of harvest with respect to the morphological trait. This was justified by Pearson correlation test which resulted in positive correlation among total morphological traits against time of harvest. The most significant correlation was observed between November 2013 and November 2014 followed by November 2015 and November 2014 as shown in Table 3 and Fig. 2. This result suggested that there is gradual increase in morphological traits over time of harvest in three consecutive years.

3.2 ISSR and RAPD markers for identification of zygotic and nucellar seedlings
ISSR markers generated the highest number of amplified fragments with an average of 7.25 bands obtained per markers (Table 4). From the total markers, only 16 ISSR and 5 RAPD primers were selected based on the polymorphic and reproducible banding patterns. Of these, finally 4 ISSRs primers namely UBC 810, UBC 835,

UBC 840, UBC 855, and 3 RAPD primers namely OPA 18, OPAA 10 and OPA 04 were selected for further analysis based on ability to differentiate DNA amplification of mother plants and seedlings from polyembryonic seeds. **UBC 855 identified as a potential marker that recorded highest polymorphism (87.5%). In case of RAPD, primer OPA 18 amplified the highest polymorphism (71.4% of the bands) with an average of 7 bands per primer**. The sizes of amplified PCR products differed among ISSR markers i.e UBC 810-700 bp, UBC 835-280 bp, UBC 840-600 bp, and UBC 855-590 bp (Fig. 3a). The three RAPD primers generated differentiating fragments of varying sizes; OPAA 10 - 650 bp, OPA 04 -710 bp, and OPA 18 - 600 bp (Fig. 3b). In addition, a distinguishable ISSR amplification pattern, related to the mother and progeny, was obtained using the UBC855 and OPAA 10 primers. The amplified UBC 855- 590 bp fragments was not from zygotic progeny but from the mother plant (Fig. 4a) and the amplified OPAA 10- 650 bp fragment was present in the zygotic progeny but not in the mother plant as indicated by arrows (Fig. 4b). Primer UBC 855 exhibited 29.0% (18/62) zygotic identification efficiency. UBC 835 primer was the second most efficient in identifying zygotic at 27.4% (17/62) (Table 4). RAPD primer OPAA 10 exhibited 41.9% (26/62) zygotic identification efficiency, followed by OPA 18 with 24.1% (15/62). Using all 4 ISSR primers, the identification rate of zygotic seedlings was 87% (54/62), while using all the 3 RAPD primers, identification efficiency was 85.4% (53/62). Using ISSR and RAPD, the zygotic identification efficiency was increased to 59.6% (37/62) when different primers were used. However, none of these primers alone were able to categorize all zygotic seedlings. This kind of observation was also reported by reported by Rajwana et al. (2008). According to Yun et al. (2007) in Miyagawaunshiu × Ponkan mandarins, 13.4% (20/149) seedlings were zygotic. Thus, a broader selection of polymorphic primers enhances the probability of identifying zygotic individuals, as cited previously by Bastianel et al. (1998).

Of 62 polyembryonic seedlings studied, 37 seedlings showed a banding pattern different from that of the mother plant using both ISSR and RAPD primers (Table 5) indicating a zygotic origin; primer UBC 855 helped to identify 18 of these zygotic seedlings (Fig. 4a) and OPAA 10 helped to identify 26 of these zygotic seedlings (Fig. 4b). A finding reported by Vilarinhos et al. (2000) identified 12 zygotic seedlings from Volkamerian lemon × Cravo lemon using six of 20 RAPD primers in a combination. Of the 7 seedlings from monoembryonic seeds, primers UBC 835 and UBC 810 identified 14.2% (1/7) and primers OPA 04, OPAA 10 and OPA 18

identified 42.8% (3/7), 28.5% (2/7) and 14.2% (1/7), respectively to be zygotic in nature (Table 5). Additionally, among both polyembryonic and monoembryonic seedlings, 53.6% of them were zygotic in nature and 46.3% nucellar, which are in close proximity to the value of 53% and 47% of zygotic and nucellar seedlings as reported by Mondal et al. (2014) in *Citrus reticulata* using RAPD. In the present study, seedlings genetically identical to the mother plant were generated at a low incidence in polyembryonic seeds produced by the highly polyembryonic population. As reported, the percentage of nucellar seedlings declines with an increase in the proportion of polyembryonic seed generated (Soost and Roose, 1996; Andrade-Rodriguez et al., 2004). Thus, it is likely that the lower proportion of nucellar population is related to a higher range of polyembryony. In the present work, 66.6% of the Khasi mandarin seeds had 2 to 3 zygotic embryos (Table 5). This coincides with Das et al. (2007) that reports when more than one zygotic embryo per seed for citrus was observed it suggests the chances of different microgametes induced fertilization.

Our result indicated that zygotic embryos were located in the micropylar region in 40% (2 of 5) of the polyembryonic seeds; while in the remaining 60% seeds, they were located near the micropyle region but not in the micropyle (Table 5). This observations suggests that embryo distribution inside seed does not follow any specific pattern and might be genetically controlled since zygotic embryos cannot be visually distinguished based on their morphological traits such as shape, size etc (Yun et al., 2007). Similar results were also observed where it was reported that the maximum of the zygotic seedlings were obtained from embryos located close to the micropylar region (Andrade-Rodriguez et al., 2005). The reason for positioning of zygotic embryos at micropylar area could be related to their growth habit which depends on the endosperm since its presence stimulates the formation of adventitious embryos in the micropylar region but suppresses their initiation towards the chalazal part based on the distance of embryo from the micropylar end (Kishore et al., 2012).

The size of embryos in polyembryonic seeds ranged from 2-6 mm, while in monoembryonic seeds, it varied from 6-8 mm (Table 5). The study indicated that embryos of even small size (2-3 mm) were able to develop seedlings suggesting the availability of food reserve as well as hydrated condition of the mature embryos during *in-vitro* seed germination. The embryo size is an important attribute in polyembryony since smaller ones are generally found to be inviable due to insufficient food reserve (Andrade-Rodriguez et al., 2005). In citrus, as the number of embryos per seed increases, its size decreases (Soares Filho et al., 2003). In orange, it was reported that

the embryo size decreases as it approaches near the micropylar region of the seed (Villegas and Andrade, 2008).

IV.   CONCLUSION

A simple outlook that emerges from the study is that the popular mandarin cultivar, Khasi mandarin is highly polyembryonic, exhibiting polyembryony in more than 80% of their seeds with more clutch size and the possibility of obtaining zygotic plants from them is high. A population of 69 individual seedlings were generated, out of which 62 individuals were tested as polyembryonic plantlets. Out of tested plantlets, 37 hybrids and 25 nucellars were recognized through molecular screening by ISSR and RAPD markers. ISSR-UBC 855 and RAPD-OPAA 10 can be used as efficient markers for identifying hybrid seedlings at an early developmental stage confirming their potential for early selection in citrus breeding. The ISSR and RAPD markers used in the present study showed 15-45% efficiency in the identification of the origin of seedlings. Observation of embryo size and position allowed us to find that zygotic seedlings were obtained from embryos located close to the micropylar region and not at the micropylar region. Since polyembryony is species and region-specific, this attempt may aid in the development of uniform population for the farmer community of the region. This work could be elaborated with a large population size or a hybrid system for overall citrus germplasm management in India.

DISCLOSURE STATEMENT
The authors declare no conflict of interest.

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Table. 1: Percentage of germination (PG), percentage of polyembryony (PP) and clutch size in Khasi mandarin[1]

Harvest	PG (%)	PP (%)	Embryos/PS*		Clutch size		No. of leaves	No. of branches	Shoot length
			Min	Max	Poly	Total			(cm)
2013	66.60±0.12a	83.30±1.0a	2	14	2.70±0.1a	2.30±0.12a	3.00±0.1a	3.00±0.12a	5.13±0.19b
2014	64.23±0.09b	55.50±1.6b	2	13	2.50±0.0ab	1.40±0.10b	4.70±0.0b	2.10±0.21b	6.00±0.21a
2015	83.37±0.09c	50.00±1.6c	2	11	2.27±0.0b	1.13±0.19b	5.20±0.2c	1.33±0.12a	6.63±0.19a

[1] Means followed by the same letter in each column are not significantly different based on Duncan's Multiple Range (DMR) test at 5% probability. Data are means ± standard error.

* Polyembryonic seeds

Table. 2: Anova analysis

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Individual	2	63	31.3	0.031	0.97
Residuals	18	18376	1020.9		

Table. 3: Pearson correlation analysis

	Nov13	Nov 14	Nov 15
Nov2013	1.0000000	0.9734644	0.9041905
Nov 2014	0.9734644	1.0000000	0.9776250
Nov2015	0.9041905	0.9776250	1.0000000

Table. 4: Status of polymorphism for Khasi mandarin using four ISSR primers and three RAPD primers

Primer	Sequence (5'-3')	Total Bands amplified	Polymorphic Bands	Polymorphism (%)	Zygotic seedlings PS[1]	MS[2]
UBC 855	(AC)8YT	8	7	87.5	18/62	0/7
UBC 835	(AG)8YC	8	5	62.5	17/62	1/7
UBC 810	(GA)9C	8	4	50.0	11/62	1/7
UBC 840	(GA)8YT	8	6	75.0	8/62	0/7
OPA 18	AGGTGACCGT	7	5	71.4	15/62	1/7
OPA 04	AATCGGGCTG	5	3	60.0	12/62	3/7
OPAA 10	TGGTCGGGTG	9	6	66.6	26/62	2/7

[1]polyembryonic seedling

[2]monoembryonic seedling

Table. 5: Zygotic (Z) or nucellar (N) origin of seedlings with respect to embryo position from polyembryonic and monoembryonic seeds analyzed using four ISSRand three RAPD primers in Khasi mandarin

Plant	Seed (no.)	Seedling (no.)	Embryo size (mm)	Position	UBC 855	UBC 835	UBC 810	UBC 840	OPA18	OPA04	OPAA10
BH1		MP			MP	MP	MP	MP	MP	MP	MP
	1	1	5	NM	N	N	N	N	N	N	N
		2	6	NM	N	Z	N	Z	N	N	N
	2	1	6	NM	N	Z	N	N	N	N	N
		2	4	NM	Z	N	N	N	N	N	N
		3	3	M	Z	N	N	Z	N	N	N
		4	4	NM	N	N	N	N	N	N	Z
	3	1	3	M	Z	Z	N	N	N	N	Z
		2	5	NM	N	N	N	Z	Z	Z	Z
		3	5	NM	N	N	Z	N	N	N	N
	4	1	4	NM	N	N	N	N	N	N	N
		2	4	NM	N	N	N	N	Z	N	N
	5	1	6	NM	N	N	N	N	Z	N	Z
		2	5	NM	N	N	N	N	N	N	Z
		3	6	NM	N	N	N	N	N	Z	N
BH2		MP			MP	MP	MP	MP	MP	MP	MP
	1	1	6	NM	Z	N	N	N	N	N	N
		2	4	NM	N	N	N	N	Z	Z	Z
	2	1	3	M	Z	Z	Z	Z	N	N	N
		2	4	NM	Z	N	N	N	N	N	N

Plant	Seed (no.)	Seedling (no.)	Embryo size (mm)	Position	UBC 855	UBC 835	UBC 810	UBC 840	OPA18	OPA04	OPAA10
	3	1	4	NM	N	N	N	N	Z	Z	Z
		2	4	NM	N	N	N	N	N	N	N
	4	1	5	NM	Z	N	N	N	N	N	N
		2	4	NM	Z	N	N	N	N	N	N
BH3		MP			MP	MP	MP	MP	MP	MP	MP
	1	1	6	NM	N	N	N	N	N	N	N
		2	3	M	N	Z	Z	Z	N	N	N
		3	6	NM	N	N	N	N	N	N	N
		4	3	M	N	N	Z	N	N	N	N
	2	1	5	NM	N	N	N	N	N	Z	Z
		2	4	NM	N	N	N	N	N	N	Z
		3	4	NM	N	N	N	N	Z	N	Z
	3	1	6	NM	N	Z	N	N	N	N	Z
		2	5	NM	N	Z	N	N	Z	N	N
	4	1	6	NM	N	N	N	N	N	Z	Z
		2	5	NM	N	Z	N	N	N	N	N
	5	1	3	M	Z	Z	N	N	N	N	Z
		2	4	NM	Z	N	N	N	N	Z	Z
BH4		MP			MP	MP	MP	MP	MP	MP	MP
	1	1	2	NM	Z	N	N	N	N	N	Z
		2	6	NM	N	N	N	N	N	N	Z
		3	6	NM	N	N	N	N	Z	N	N
	2	1	5	NM	N	N	N	Z	N	Z	N
		2	3	M	Z	N	Z	N	N	Z	N
	3	1	6	NM	N	N	N	N	N	N	N
		2	5	NM	N	Z	N	N	N	N	Z
		3	5	NM	N	Z	N	N	N	N	Z
	4	1	6	NM	N	N	N	N	Z	N	Z
		2	3	M	Z	Z	N	Z	N	N	Z
BH5		MP			MP	MP	MP	MP	MP	MP	MP
	1	1	4	NM	Z	N	N	N	N	N	Z
		2	6	NM	N	N	N	N	N	Z	Z
	2	1	5	NM	N	N	Z	N	Z	Z	N
		2	6	NM	N	N	N	N	Z	N	N
		3	5	NM	Z	N	N	N	N	N	N
	3	1	4	NM	N	Z	N	N	N	N	N
		2	3	M	Z	Z	N	N	N	N	N
	4	1	4	NM	N	N	N	Z	Z	N	N
		2	3	M	N	N	Z	N	Z	N	N
	5	1	5	NM	N	Z	Z	N	N	N	N
		2	4	NM	Z	Z	N	N	N	N	N
BH6		MP			MP	MP	MP	MP	MP	MP	MP
	1	1	6	NM	N	N	N	N	N	N	Z
		2	5	NM	N	N	Z	N	Z	N	Z
	2	1	5	NM	N	N	N	N	N	N	Z
		2	4	NM	Z	Z	Z	N	N	N	Z
	3	1	6	NM	N	N	Z	N	N	N	N
		2	5	NM	N	N	N	N	Z	Z	N
	M1	1	7	-	N	N	N	N	N	N	N
	M2	1	8	-	N	N	N	N	N	N	N

Plant	Seed (no.)	Seedling (no.)	Embryo size (mm)	Position	UBC 855	UBC 835	UBC 810	UBC 840	OPA18	OPA04	OPAA10
	M3	1	6	-	N	N	N	N	N	N	N
	M4	1	6	-	N	N	N	N	Z	N	Z
	M5	1	8	-	N	N	N	N	N	Z	N
	M6	1	7	-	N	Z	N	N	N	Z	N
	M7	1	8	-	N	N	Z	N	N	Z	Z

MP: mother plant; NM: non-micropylar; M: micropylar; N: nucellar; Z: zygotic; M1-M7: monoembryonic seedling; -: not applicable

Fig. 1: [a] (1, 2): Variation in partitioning of embryos formed in a seed from the polyembryonic Khasi mandarin cultivar, (3): Embryos at micropylar region, (4): Embryo from monoembryonic Khasi mandarin cultivar. Bar in [a] (1, 3, 4) is 3 cm and [a] (2) is 2 cm; [b] (1, 2, 3): Asynchronous appearance of triplet seedlings in Khasi mandarin, (4): Synchronous appearance of duplet seedlings in Khasi mandarin. Bar in [b] (1) is 0.5 cm and [b] (2, 3, 4) is 1 cm
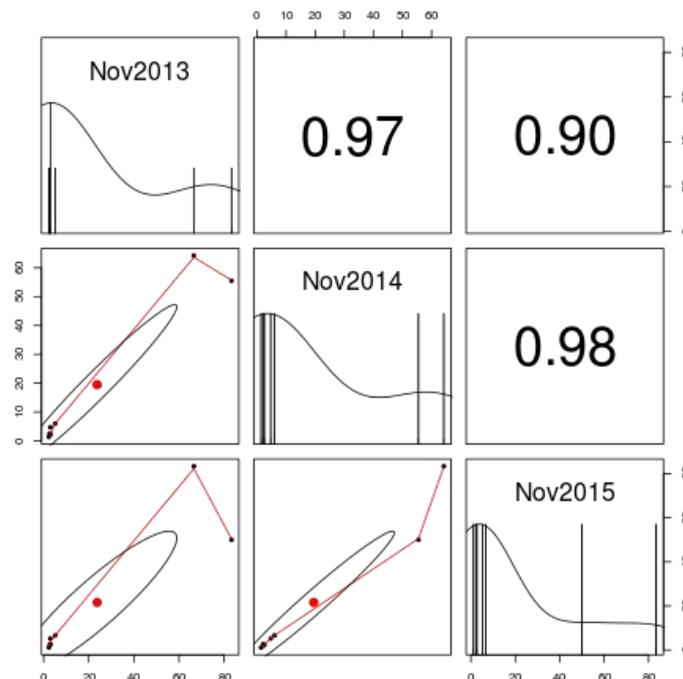

Fig. 2: Significant values among total morphological traits against time of harvest pass through positive correlation baseline as depicted through Pearson correlation test.
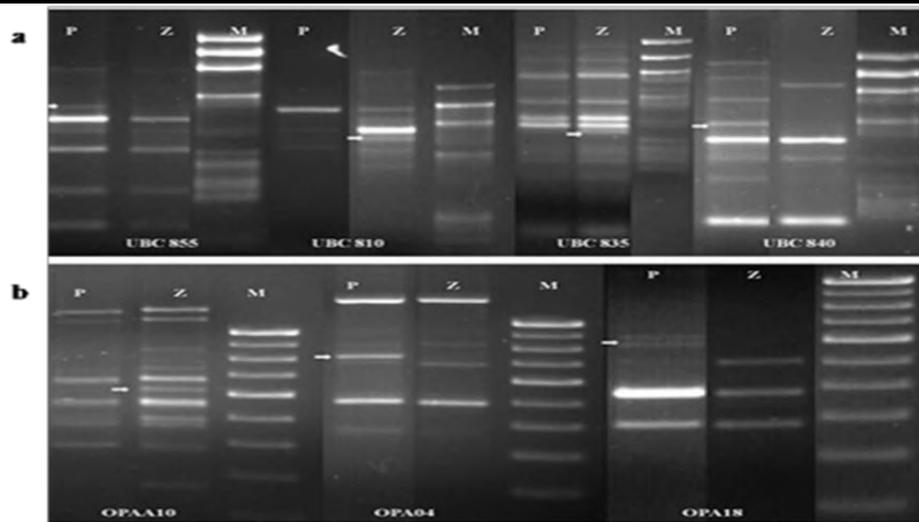
Fig. 3: (a)Mother-progeny related DNA amplification patterns showing polymorphism by using four screened ISSR primers (UBC855, UBC 810, UBC 835, and UBC 840);(b)using three RAPD primer (OPAA10, OPA04, and OPA 18). The white arrowheads indicate the above mentioned confirmed polymorphic markers that can be used for the identification of hybrid seedlings with a zygotic origin. P: mother; Z: seedling with zygotic origin; M (a) ØX/ HaeIII digest and M (b) is 1 Kb DNA ladder
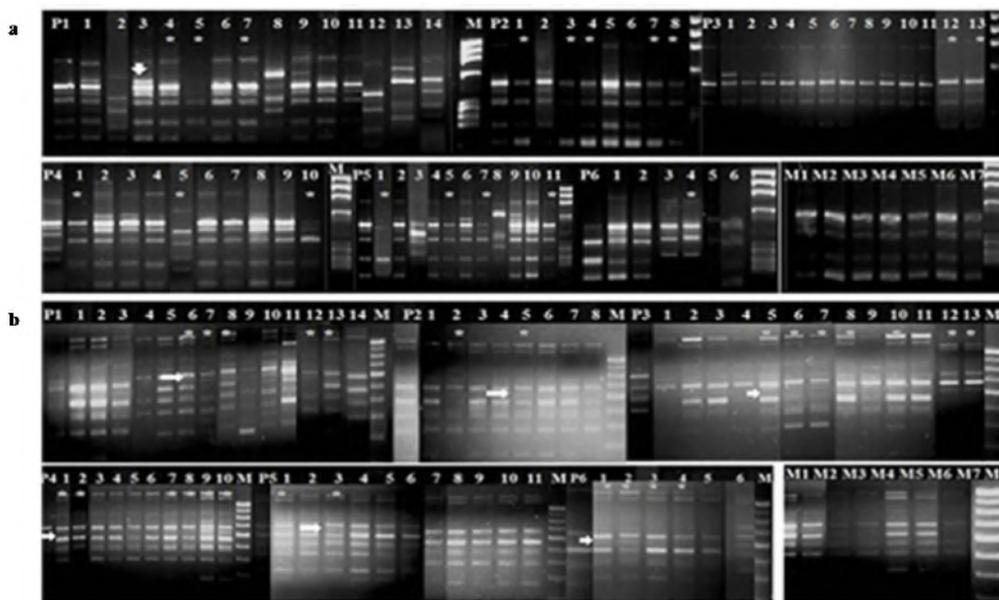

Fig. 4: (a) Putative zygotic seedlings were identified by ISSR marker UBC 855; (b) identified with RAPD marker OPAA 10. The white arrowheads indicate a 590 bp in upper one (total 18 putative zygotic seedlings) and a 650 bp in lower one (total 26 putative zygotic seedlings). M (a) ØX/ HaeIII digest and M (b) is 1 Kb DNA ladder; P1-P6: six mother trees; numerals after P1, P2, P3, P4, P5 and P6 represents polyembryonic seedling with zygotic or nucellar origin from each mother plant respectively; M1-M7: monoembryonic seedlings