ARTICLE

Selection of SSR markers for identifying embryonic origin in three polyembryonic mango rootstock varieties

Crop Breeding and Applied Biotechnology
26(2): e54582628, 2026
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332026v26n2a23

Alessandro Rosa Nascimento[1], Vespasiano Borges de Paiva Neto[1], Antonio Elton da Silva Costa[2], Kecia Mayara Galvão de Araújo[1], Jerônimo Constantino Borel[1] and Francine Hiromi Ishikawa[1]*

Abstract: *Polyembryonic mango varieties, often used as rootstocks, produce two or more embryos per seed, one of which is genetically distinct from the mother plant. This study aimed to differentiate three mango varieties regarding embryonic origin, vigor, and genetic non-uniformity using SSR markers. The mango varieties evaluated were Espada, Capucho, and Coquinho, which are commonly used as rootstocks. Thirty primers reported in the literature were tested, along with variables such as emergence percentage, seedling number, and extent of polyembryony. Only primers MillHR26, MillHR36, MillHR32, and MillHR28 were polymorphic. No direct association was found between seedling vigor and embryonic origin. Zygotic embryos may have been suppressed in the Capucho variety, which also exhibited a greater extent of polyembryony. This probable suppression, combined with higher polyembryony, indicates Capucho as the most suitable rootstock source for achieving genetic uniformity.*

Keywords: *Polyembryony, SSR molecular markers, rootstock variety*

INTRODUCTION

Mango exhibits high global economic importance, and in 2022, it ranked among the three most traded tropical fruits worldwide. According to FAO (2023), in 2023, the production of mango, mangosteen, and guava exceeded 60 million tons, with Brazil ranking sixth among the world's largest producers. In 2023, the country produced more than 1.7 million tons of mangoes and an estimated production value of over 3.2 billion reais, with emphasis on the Sub-middle São Francisco valley region (IBGE 2023). Mango cultivation is highly relevant, both economically and in terms of job creation for this region of Brazil (Gazzola et al. 2020).

In Brazil, both polyembryonic and monoembryonic mango varieties are cultivated. In polyembryonic varieties, the seeds contain two or more embryos, one of which can be zygotic, while the others have nucellar origin. The monoembryonic variety, however, has only a zygotic embryo (Kalal et al. 2023). Due to this, to preserve the agronomic characteristics of monoembryonic varieties, vegetative propagation is employed in commercial orchards, grafting the scion cultivars onto polyembryonic rootstocks (Jain et al. 2025).

***Corresponding author:**
E-mail: francine.hiromi@univasf.edu.br

Scientific Editor:
Luiz Antônio dos Santos Dias

Received: 10 November 2025
Accepted: 18 March 2026
Published: 09 April 2026

[1] Universidade Federal do Vale do São Francisco (UNIVASF), Colegiado de Engenharia Agronômica, Rodovia BR-407, km 12, Lote 543, s/n, 56300-000, Petrolina, PE, Brazil
[2] Universidade Federal Rural de Pernambuco (UFRPE), Departamento de Agronomia, Rua Dom Manoel de Medeiros, s/n, 52171-900, Recife, PE, Brazil

In the Northeast region of Brazil, with emphasis on the Sub-middle São Francisco Valley, the mango (*Mangifera indica* L.) varieties Espada, Capucho, and Coquinho are adapted to local edaphoclimatic conditions and are used as rootstocks in mango seedling production (Santos et al. 2024). Despite their widespread use, studies focusing on these varieties remain scarce, particularly those employing molecular biology tools aimed at the characterization and their genetic variation.

In mango breeding, the zygotic embryo is of interest, because it gives rise to plants genetically distinct from their parental lines. However, for commercial seedling production, growers focus on those derived from the nucellar embryo, which are considered clones of the female parent, providing greater genetic uniformity among the seedlings (Rocha et al. 2014, Jain et al. 2025).

In order to ensure this genetic uniformity, seedlings originating from the zygotic embryo should be discarded. For this purpose, it is common practice among seedling producers to perform phenotypic selection based on vigor, considering the more vigorous seedlings as originating from the nucellar embryo (Kalal et al. 2023).

However, it is difficult to differentiate zygotic from nucellar seedlings based on vigor. Rocha et al. (2014), working with the cultivar 'Rosinha', when evaluating the polyembryonic genotype of mango UBÁ using ISSR markers, found zygotic seedlings in 18 seeds, six of which were among the most vigorous. They concluded that, for this genotype, the more vigorous plants may be of zygotic origin. Cordeiro et al. (2006) studied the relationship between embryo position, vigor, and embryonic origin in the cultivar 'Rosinha' and observed that the zygotic embryo could occupy different positions within the seed, and that 90% of the most vigorous seedlings were of zygotic origin. Marcial et al. (2021), working with the polyembryonic cultivar 'Ataulfo', found that 15% of the largest embryos in the seed gave rise to zygotic seedlings. Factors such as these can reduce the effectiveness of methods based on traditional visual morphological assessments to distinguish zygotic seedlings due to the overlap of characteristics between zygotic and nucellar embryos (Tardivo et al. 2025). This can consequently promote variability among seedlings, potentially reducing orchard production performance.

The use of molecular markers allows for more accurate identification of genetic variations among individuals. Among the molecular markers that can be used for this purpose, SSRs (Simple Sequence Repeats) stand out, as they have been frequently employed due to their high degree of polymorphism, codominance, and high informativeness, being widely applied in studies of genetic diversity, parentage analysis, genetic linkage, evolutionary history, among others (Srivastav et al. 2021, Sridhar et al. 2022, Koltun et al. 2024).

Thus, the objective of this study was to select polymorphic SSR loci through a screening of primers available in the literature to differentiate three mango varieties regarding embryonic origin, vigor, and genetic non-uniformity using SSR markers. The mango varieties evaluated were Espada, Capucho, and Coquinho, which are commonly used as rootstocks.

MATERIAL AND METHODS

Primer selection from the literature

In this study, 30 primers were tested and selected based on their polymorphic information content (PIC), as reported in the studies conducted by Ravishankar et al. (2011), Ravishankar et al. (2015), Ravishankar et al. (2017) and Alves et al. (2016). The linkage group of the SSR loci was also considered during the selection process, prioritizing primers from different groups in order to ensure broader genetic coverage of the individuals.

Primer screening

For primer screening, DNA was extracted from leaves of 12 mango seedlings, four seedlings per rootstock variety (Espada, Capucho, and Coquinho). The seedlings were obtained from certified nurseries located in the municipalities of Petrolina, Pernambuco, and Curaçá, Bahia, Brazil.

DNA extraction was carried out using a modified 2X CTAB protocol, originally adapted for mango (*Mangifera indica* L.), as described by Lima et al. (2007), combined with the modified method of Ferreira and Grattapaglia (1995). Modifications included the integration of steps from both protocols, such as the use of steel beads, absent in the protocol of Lima et al. (2007), and the addition of pre-heated (60 °C) CTAB buffer directly to microtubes containing the plant tissue, followed by maceration without returning the samples to the water bath.

Fully expanded young leaves from mango seedlings were collected, labeled, placed in separate plastic bags, and transported to the Laboratory of Plant Pathology and Breeding at the Federal University of Vale do São Francisco (UNIVASF), Agricultural Sciences Campus, Petrolina-PE. Between 100 and 150 mg of leaf tissue were weighed and transferred to 1.5 mL microtubes containing four 2-mm stainless steel beads. To this material, 950 µL of CTAB buffer was added, previously prepared with the following composition: 0.2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 500 mM Tris-HCl (pH 8.0), and 2% β-mercaptoethanol, pre-heated to 60 °C for 10 min. Samples were then macerated using an L-Beader 3 disruptor at 4,000 rpm for 4 min.

The samples were cooled at room temperature for 5 min, followed by the addition of 600 µL of chilled CIA solution (Chloroform:Isoamyl Alcohol, 24:1) for protein precipitation, and centrifuged at 4,000 rpm for 10 min. The aqueous phase was pipetted four times in aliquots of 100 µL, transferred to new 1.5 mL microtubes, and mixed with 400 µL of isopropanol (–20 °C) for DNA precipitation. Tubes were kept on ice for 10 min, and when dark precipitates were observed at the bottom, the supernatant was carefully removed. Samples were then centrifuged for 10 min at 4,000 rpm, and the supernatant was discarded.

The resulting pellet was air-dried at room temperature for 1 h to allow complete ethanol evaporation, followed by the addition of 30 µL of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing 10% RNase. Samples were incubated at 37 °C for 45 min. DNA integrity was assessed by electrophoresis in 2% agarose gel at 80 V for 1 h. Purity was evaluated by the 260/280 ratio, and concentration (ng μL^{-1}) was determined using a NanoDrop Lite 1000 spectrophotometer.

DNA samples were subsequently used in polymerase chain reaction (PCR). Each PCR reaction was performed in a final volume of 20 µL containing 150 ng of genomic DNA, 2 µL of 10X buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM forward and reverse primers, and 0.4 µL of Taq DNA polymerase. Thermal cycling conditions consisted of an initial denaturation at 94 °C for 1 min, followed by extension at 72 °C for 1 min. This was followed by 35 cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

Amplification products were first evaluated by electrophoresis in 2% agarose gel at 80 V for 1 h. Primers that produced amplification in all individuals were Polymorphic primers: MillHR26 (Forward: GCGAAAGAGGAGAGTGCAAG, Reverse: TCTATAAGTGCCCCCTCACG), MillHR36 (Forward: TCTATAAGTGCCCCCTCACG, Reverse: ACTGCCACCGTGGAAAGTAG), MillHR32 (Forward: TGGTGGTGTTTGTTTGCAGT, Reverse: ACCACCCGCAGTATTGAAAG), and MillHR28 (Forward: GCGGTCGCAGACAAATTCTATAT, Reverse: ACAACTCGAGATTGTCACATCTTT). Then, they were subjected to 6% polyacrylamide gel electrophoresis for 3 h and visualized with silver nitrate staining to assess polymorphism. Primers that failed to amplify in two or more individuals of the same rootstock variety were discarded.

Emergence percentage, polyembryony extent, and average number of seedlings per seed in each rootstock variety

The rootstock varieties were also evaluated for emergence percentage, polyembryony extent, and the average number of seedlings per seed. For this purpose, ten fruits of the Espada, Capucho, and Coquinho varieties were randomly collected from mango trees in Petrolina, Pernambuco, and Juazeiro, Bahia, Brazil. Seeds were extracted from the endocarp and a week later they were sown in plastic bags (17 cm wide × 22 cm high) filled with a mixture of Vertisol and sand (1:1, v/v), arranged at 0.2 × 0.2 m spacing in a completely randomized design. Seedlings were irrigated daily in the late afternoon using a watering can.

Emergence percentage (EM%) and polyembryony extent (E%) were calculated according to Kumar et al. (2020), with a modification in the formula for polyembryony extent. The modification consisted of using the total number of emerged seedlings instead of the number of seeds that produced more than one seedling.

$$EM\ (\%) = \frac{Number\ of\ emerged\ seeds}{Number\ of\ sown\ seeds}$$

$$E\ (\%) = \frac{Total\ number\ of\ emerged\ seedlings}{Number\ of\ emerged\ seeds}$$

The data for emergence percentage, polyembryony extent, and average number of seedlings per seed were numerically evaluated. Thirty days after sowing, the number of germinated seeds and the number of seedlings per seed were recorded.

Molecular evaluation of the relationship between seedling vigor and embryonic origin

For molecular analysis, 30 days after sowing, leaf DNA was extracted from seedlings obtained from five seeds of each rootstock variety. Samples were labeled with a three-digit code to identify the variety, the seed, and the individual seedling from which the sample was collected: the first digit represented the rootstock variety (1 – Espada, 2 – Capucho, and 3 – Coquinho); the second digit indicated the seed (ranging from 1 to 5); and the third digit indicated the seedling, depending on the number of seedlings obtained per seed. The DNA sample of the most vigorous individual was marked with an asterisk (*). The most vigorous individual was defined as the one showing greater development in terms of height and number of leaves, assessed visually. In this step, during polyacrylamide gel electrophoresis, a control DNA sample of each rootstock variety was added, which was previously characterized as nucellar during primer screening.

Molecular data analysis

Molecular markers that revealed distinct banding patterns within the studied population were considered polymorphic. Seedlings were grouped according to their embryonic origin into nucellar and zygotic, based on the observed banding patterns. Seedlings were classified as nucellar when they exhibited identical banding patterns among individuals of the same rootstock variety for all primers evaluated in the electrophoresis analysis. Conversely, seedlings were classified as zygotic when they showed different banding patterns for at least one of the markers used. Based on molecular data from seedlings evaluated for the relationship between seedling vigor and embryonic origin, the Probability of Identity (PI) and the Probability of Identity among siblings (PIsibs) were estimated using GenAlEx 6 (Nordlander et al. 2022).

RESULTS AND DISCUSSION

Primer screening

Of the 30 primers evaluated, 12 were discarded during agarose gel evaluation due to amplification problems in Capucho and/or Coquinho rootstocks, amplifying in few or none of the individuals. All primers successfully amplified the individuals of Espada.

Of the 16 remaining primers subjected to polymorphism analysis on polyacrylamide gels, four were polymorphic (MillHR26, MillHR36, MillHR32, and MillHR28), amplifying one to five alleles in the region between 100 and 500 bp, and allowing the identification of the embryonic origin of seedlings in the Capucho and Coquinho rootstocks. In contrast, no polymorphisms were observed in the Espada variety (Table 1; Figure 1). All primers classified as polymorphic enabled differentiation among the studied varieties (Figure 1).

The absence of polymorphism observed in individuals of the Espada rootstock may be related to the lack of genetic variability among individuals due to their nucellar origin. In polyembryonic mango genotypes, among the multiple seedlings derived from a single seed, one may be of zygotic origin while the others are nucellar, resulting in low genetic variability and the production of seedlings that maintain the characteristics of the mother plant (Viruel et al. 2005, Song et al. 2023, Ali et al. 2025). Therefore, even seedlings originating from different seeds and mother plants may have the same genetic constitution due to the prevalence of clonal individuals within the population.

Using primer MillHR26, two alleles were amplified in all individuals in the region between 100 and 200 bp, except for individual C3, for which only one allele was amplified. Consequently, this individual and Co3 were characterized as

Table 1. Differentiation of mango seedlings from different rootstocks regarding embryonic origin as zygotic (Z) or nucellar (N) using SSR markers. Seedlings of Espada (E), Capucho (C), and Coquinho (Co)

Primers SSR	ESPADA				CAPUCHO				COQUINHO			
	Individual											
	E1	E2	E3	E4	C1	C2	C3	C4	Co1	Co2	Co3	Co4
MillHR26	N	N	N	N	N	N	Z	N	N	N	Z	N
MillHR36	N	N	N	N	N	N	N	N	N	N	Z	N
MillHR32	N	N	N	N	N	N	Z	N	N	N	N	Z
MillHR28	N	N	N	N	N	N	Z	N	N	N	Z	N

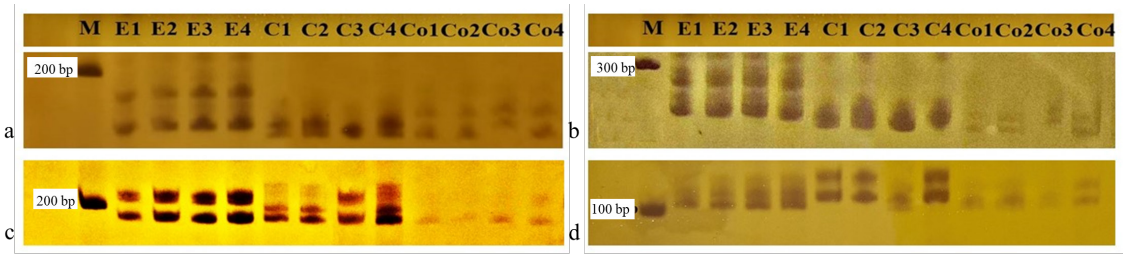

Figure 1. Banding patterns for the primers SSR in the primer screening stage: a) MillHR26; b) MillHR36; c) MillHR32 and, d) MillR28. The rootstocks evaluated were represented by Espada (E); Capucho (C); and Coquinho (Co). Bp = base pairs.

zygotic in origin due to their distinct banding patterns compared to others of the same rootstock variety. This primer also allowed clear differentiation among the rootstock varieties based on the separation pattern of the amplified alleles, with the greatest separation observed for Espada, Coquinho, and Capucho individuals, respectively (Figure 1a).

For primer MillHR36, two alleles were amplified in Espada and Coquinho individuals in the region between 200 and 300 bp. Similar to what was observed with MillHR26, individual Co3 was distinct from the other Coquinho individuals and confirmed as zygotic in origin. This primer also revealed distinct molecular profiles among the rootstock varieties. In Espada, a greater separation between amplified alleles was observed, whereas in Coquinho, the separation was smaller. For Capucho, a single allele was amplified in all individuals, characterizing them as homozygous at this locus (Figure 1b).

With primer MillHR32, two alleles with identical banding patterns were amplified in the 100–300 bp region for Espada (E1, E2, E3, E4) and Capucho (C1, C2, C4) individuals. Although two alleles were also amplified for individual C3 in the same region, the banding pattern differed from the other individuals of this rootstock, thus classifying it as zygotic, similar to the result observed with MillHR26 (Figure 1c).

For Coquinho, a single allele was amplified for all individuals except C4, for which two alleles were amplified, classifying it as a zygotic heterozygote. The molecular profile among the varieties was also clearly distinct for this primer, with greater allele separation observed for Espada and Capucho individuals, respectively (Figure 1c).

For primer MillHR28, two alleles were amplified in the 100-200 bp region for all individuals, except for C3 and Co3, which amplified only one allele, differing from individuals of their respective varieties and confirming the results obtained with MillHR26 and MillHR32 for C3 and MillHR26 and MillHR36 for Co3. This primer also allowed clear distinction of Capucho individuals from the other rootstocks, due to the largest observed separation between amplified alleles among the varieties (Figure 1d). The remaining primers were discarded due to monomorphic behavior in electrophoresis, nonspecific band formation, or unreliable banding profiles, preventing accurate molecular analysis of the rootstock varieties.

In this study, although 30 highly polymorphic primers were selected from the literature, only 16 amplified all individuals, and only four demonstrated polymorphisms. The absence of polymorphism in 11 primers may be explained by differences between the populations studied by Ravishankar et al. (2011), Ravishankar et al. (2015), Ravishankar et al. (2017) and Alves et al. (2016) and the population in this study, where even among studies by the same authors, variation in the number of alleles and the polymorphic information content (PIC) for the same molecular marker is observed, although none had a PIC value below 0.5.

According to Yan et al. (2024), the polymorphic information content of a primer is a crucial factor in determining its utility and reflects how informative a primer can be for a given population, with values ranging from 0 to 1. Primers with values above 0.5 are considered highly informative. Therefore, identifying and using polymorphic primers is essential for detecting genetic polymorphisms and differentiating individuals.

Supporting the results obtained by Kumar et al. (2020), it was observed that using a higher number of molecular markers can increase the accuracy of individual differentiation. For example, the zygotic origin of individual C4 was only detected with primer MillHR32, whereas other individuals, such as C3 and Co3, had their origin confirmed using more

than two primers. According to Ferreira and Grattapaglia (1998), each SSR primer represents a single genetic locus in the genome. Therefore, using a greater number of SSR markers allows broader coverage of specific genome regions, providing higher reliability in the results obtained.

Molecular evaluation of the relationship between seedling vigor and embryonic origin

The results obtained during the screening phase for the four primers considered polymorphic were subsequently confirmed during the molecular evaluation of seedlings derived from the five seeds, where the same allele separation pattern was observed for each of the studied rootstock varieties in plants characterized as nucellar, confirming that these primers can be used to differentiate seedlings of the Capucho, Coquinho, and Espada rootstocks (Figure 2).

Primers MillHR26, MillHR36, and MillHR32 enabled the identification of the embryonic origin of seedlings in Espada, Coquinho, and Capucho varieties (Table 2). Primer MillHR26 allowed the identification of the highest number of zygotic individuals (Espada 25%; Capucho 0%; Coquinho 16.66%), followed by primers MillHR32 (Espada 0%; Capucho 0%; Coquinho 33.33%) and MillHR36 (Espada 12.5%; Capucho and Coquinho 0%). No zygotic individuals were identified with primer MillHR28. Among the studied varieties, Coquinho exhibited the highest proportion of zygotic seedlings (50%).

In the Espada variety, two seeds produced a single seedling, and in seed 3, which produced two seedlings, no difference in vigor was observed between individuals. In seed 4, both the most vigorous and the less vigorous individuals were of nucellar origin, whereas in seed 5, the most vigorous individual was nucellar, and the less vigorous exhibited zygotic origin (Table 2; Figure 2).

In the Capucho variety, a higher number of seedlings per seed was obtained (ranging from two to three seedlings per seed); however, all were classified as nucellar (Table 2; Figure 2). The absence of zygotic seedlings in this variety may be related to a mechanism suppressing zygotic seedling development. Rocha et al. (2014) reported that, in polyembryonic cultivars, zygotic seedlings are typically located near the basal region and may degenerate due to competition with

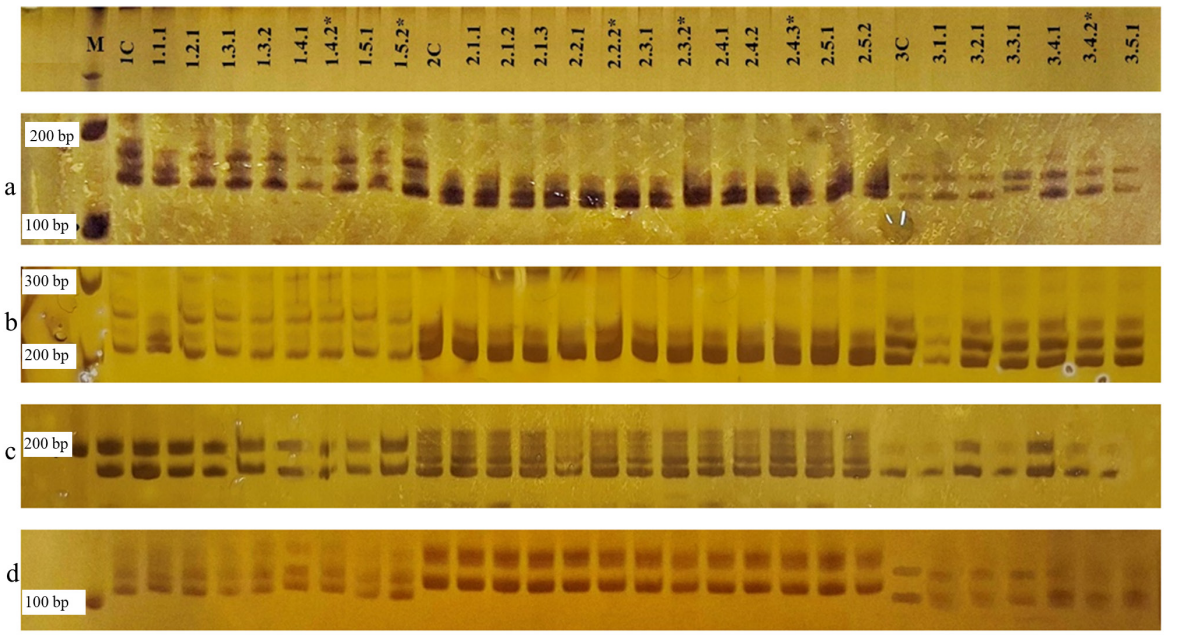

Figure 2. Banding patterns for the primers SSRs in the primer screening stage: a) MillHR26; b) MillHR36; c) MillHR32 and, d) MillHR28. Samples indicated by the letter 'C' corresponding to control, that is, the DNA of individuals characterized as nucellar for each evaluated rootstock variety. The first digit refers to the rootstock variety (1 – Espada; 2 – Capucho; 3 – Coquinho); the second digit represents the seed from which the individual originated; and the third digit represents the specific individual from that seed. Bp-base pairs. *Indicates the most vigorous seedling.

Table 2. Differentiation of mango individuals in the different rootstocks according to embryonic origin as zygotic or nucellar using SSR markers. *VAR-Variety; S.I.-Seedling individual; C – Control sample previously characterized as nucellar. *Indicates the most vigorous seedling*

VAR.		ESPADA										CAPUCHO														COQUINHO						
SEED			1	2	3		4		5			1		2		3		4		5			1	2	3		4		5	
S. I.		C	1	1	1	2	1	2 *	1	2 *	C	1	2	3	1	2 *	1	2 *	1	2	3 *	1	2	C	1	1	1	1	2 *	1
MARKERS	MillHR26	N	Z	N	N	N	N	N	Z	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N
	MillHR36	N	Z	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	MillHR32	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	Z	N	N
	MillHR28	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

nucellar embryos. Another possible mechanism is stenospermocarpy, a physiological disorder in which fertilization occurs, but the zygotic embryo degenerates before reaching physiological maturity (Navarro et al. 2023).

In this study, although the nucellar embryonic origin coincided with the most vigorous seedling in one seed of both Espada and Coquinho, it is not possible to establish a definitive relationship between vigor and embryonic origin. Seed emergence in both varieties was low (five seeds in Espada and five in Coquinho), allowing analyses to be conducted using only five seeds per variety. Additionally, some seeds produced only a single individual, preventing comparison of vigor among seedlings. In the Capucho variety, all seedlings were nucellar.

Regarding the PI and PIsibs analyses performed, both values decreased as the number of markers increased. Using one SSR, the PI values obtained were 0.30, 0.38, and 0.29; with two SSRs, 0.09, 0.38, and 0.11; with three SSRs, 0.03, 0.14, and 0.04; and with four SSRs, 0.01, 0.05, and 0.02 for the Espada, Capucho, and Coquinho rootstocks, respectively. The PIsibs values obtained were: using one SSR, 0.55, 0.59, and 0.54; with two SSRs, 0.30, 0.59, and 0.32; with three SSRs, 0.18, 0.35, and 0.19; and with four SSRs, 0.11, 0.21, and 0.11 for the Espada, Capucho, and Coquinho rootstocks, respectively (Figure 3). According to Nordlander et al. (2022), PI measures the probability that two randomly selected samples exhibit identical SSR profiles by chance, whereas PIsibs, provides a more conservative estimate by considering the possibility of identity among siblings. The decrease in PI and PIsibs values as the number of markers increases corroborates the findings reported by Yan et al. (2023), in which increasing the number of molecular markers led to PI and PIsibs values tending to zero. Low PI values indicate high fingerprinting power (Zhang et al. 2024).

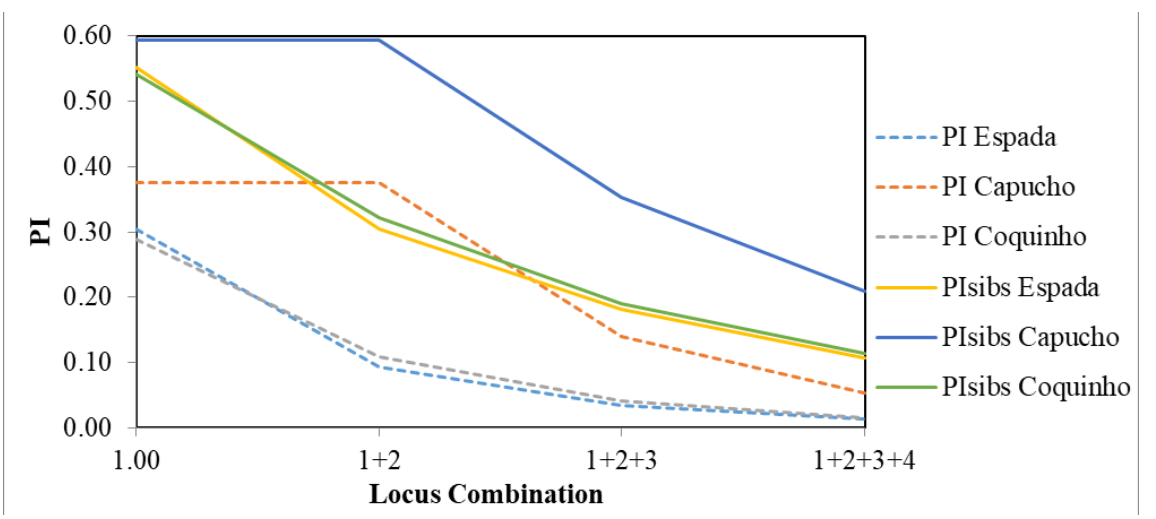

Figure 3. Probability of Identity (PI) and Probability of Identity among siblings (PIsibs) as a function of increasing locus combinations estimated using GenAlEx 6 for the three rootstocks (Espada, Capucho, and Coquinho).

Seedling emergence percentage and polyembryony extent in each rootstock variety

The highest emergence percentage was observed in the Capucho variety (100%), followed by Coquinho and Espada, both of which exhibited 50% emergence. Regarding the extent of polyembryony, the highest values were observed in Capucho, Espada, and Coquinho, with 260%, 150%, and 120%, respectively.

Kumar et al. (2020), similarly, obtained variable values for emergence percentage and polyembryony extent when studying polyembryonic rootstocks of the 13-1, Kurukan, and Ollour varieties, attributing these variations to the genotypes themselves and to genotype–environment interactions.

The likelihood of obtaining nucellar rootstocks increases in varieties with polyembryony extent greater than 80% (Kumar et al. 2020). However, in the formula proposed by these authors, polyembryony extent is calculated based on the number of seeds producing more than one seedling, disregarding the total number of seedlings produced. Consequently, different varieties may present the same polyembryony extent but produce different numbers of seedlings. To account for the number of seedlings produced per variety, the formula was modified in this study to allow the selection of varieties that not only produce more seeds with multiple seedlings but also generate a higher total number of seedlings.

The average number of seedlings obtained for Capucho, Espada, and Coquinho was 2.6, 1.5, and 1.2, respectively. Among the studies consulted, only Rocha et al. (2014) reported the occurrence of more than one zygotic embryo per mango seed. Therefore, the use of varieties that produce a higher number of seedlings per seed for rootstock production may reduce genetic variability among seedlings by increasing the proportion of nucellar to zygotic seedlings. In this context, based on polyembryony extent and the number of seedlings per seed, the Capucho variety would be the most suitable for producing rootstocks aimed at reducing genetic variability among seedlings.

CONCLUSIONS

The primers MillHR26, MillHR36, MillHR32, and MillHR28 can be applied both for the identification of hybrids and for differentiation of the Espada, Capucho, and Coquinho varieties. It was not possible to confirm a relationship between embryonic origin and seedling vigor; further studies with a larger experimental sample are necessary. Zygotic embryos in the Capucho variety appeared to have their development suppressed in all seeds evaluated. The Capucho variety proved to be the most suitable for obtaining genetically uniform rootstocks, considering its higher polyembryony extent, germination percentage, and number of seedlings obtained per seed.

AKNOWLEDGEMENTS

We are thankful for the support of the Graduate Studies Program in Plant Production (Programa de Pós-Graduação em Produção Vegetal - PPGA-PV) of the Universidade Federal do Vale do São Francisco (UNIVASF). This study was funded by Coordination for the Improvement of Higher Education Personnel (CAPES), and the Foundation for Support to Science and Technology of the State of Pernambuco (FACEPE).

CREDIT STATEMENT

Conceptualization: Nascimento AR, Neto VBP, Costa AES, Ishikawa FH; Supervision: Ishikawa FH, Neto VBP, Costa AES; Methodology and Molecular data analysis: Nascimento AR, Ishikawa FH; Investigation: Nascimento AR, Borel JC, Araújo KMG; Writing and Review: Nascimento AR, Ishikawa FH. All authors critically revised the manuscript and approved of the final version.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current research are available from the corresponding author upon reasonable request.

REFERENCES

Ali GS, Eltaher S, Li J, Freeman B and Singh S (2025) GWAS identifies a polyembryony locus in mango: development of KASP and PACE markers for marker-assisted breeding. **Frontiers in Plant Science 16**: 1-15.

Alves EOS, Lima Neto FPL, Santos CAF, Ribeiro ICNS, Melo CAF, Holanda ISA, Souza AP and Corrêa RX (2016) Genetic diversity of mango accessions (*Mangifera indica*) using new microsatellite markers and morphological descriptors. **Australian Journal of Crop Science 10**: 1281-1287.

Cordeiro, MCR, Pinto ACQ, Ramos VHV, Faleiro FG and Fraga LMS (2006) Identificação da origem genética de plântulas em sementes poliembriônicas de mangueira (*Mangifera indica* L.) cv. Rosinha por meio de marcadores RAPD. **Revista Brasileira de Fruticultura 28**: 454-457.

FAO - Food and Agriculture Organization of the United Nations (2023) FAOSTAT: Produção agrícola de cultivos e produtos pecuários. Avaiable at <https://www.fao.org/faostat/en/#data/QCL>. Acessed on August 11, 2025.

Ferreira ME and Grattapaglia D (1995) Protocolo de extração de DNA modificado. Avaiable at <https://www.ufpe.br/documents/742645/0/Protocolo_de_Extra%C3%A7%C3%A3o_de_DNA_Ferreira_e_Grattapaglia.pdf/6c5ac917-207e-4ab2-956a-6be5918d7ff5>. Acessed on January 11, 2024.

Ferreira ME and Grattapaglia D (1998) **Introdução ao uso de marcadores moleculares em análises genéticas**. Embrapa, Brasília, 220p.

Gazzola R, Gründling RDP and Aragão AA (2020) Produção e mercado internacional de manga. **Revista Brasileira de Agrotecnologia 10**: 81-87.

IGBE - Instituto Brasileiro de Geografia e Estatística (2023) Produção agrícola municipal: Brasil e unidades da Federação. Table 1613. Avaiable at <https://sidra.ibge.gov.br/tabela/1613#resultado>. Acessed on August 11, 2025.

Jain S, Prakash J, Singh SK, Kumar C, Srivastav M, Singh K, Pandey R, Sharma S, Singh A and Kumari (2025) Influence of different rootstock-interstock-scion combinations on mango (*Mangifera indica* L.) traits. **Frontiers in Plant Science 16**: 1625932.

Kalal P, Nandeesha P, Sane A, Kurian RM, Sanakaran M and Sriram S (2023) Microsatellite markers based identification of zygotic and nucellar embryos in polyembryonic mango (*Mangifera indica* L.) genotypes Vellaikulamban and Olour. **Indian Journal of Plant Genetic Resources 36**: 227-232.

Koltun A, Silva PA, Torres IY, Bonifácio-Anacleto F and Yassitepe JECT (2024) Microsatellite markers in maize: challenges and guidelines for implementing multiplex SSR analyses. **Crop Breeding and Applied Biotechnology 24**: e46392411.

Kumar K, Srivastav M, Singh KS and Singh A (2020) SSR marker based differentiation of zygotic and nucellar seedlings in mango (*Mangifera indica*). **The Indian Journal of Agricultural Sciences 90**: 2101-2107.

Lima RSN, Santos CAF, Rodrigues MA and Batista PP (2007) Ajustes no protocolo de DNA CTAB 2x para diversas espécies vegetais. Avaiable at <https://www.embrapa.br/busca-de-publicacoes/-/publicacao/160442/ajustes-no-protocolo-de-dna-ctab-2x-para-extracao-de-dna-em-diversas-especies-vegetais>. Acessed on February 11, 2024.

Marcial LCM, Ángel VM, H AZM and Serafin CI (2021) Identification of zygotic and nucellar seedlings, originated by the largest embryo in mango seeds cv Ataulfo, using simple-sequence repeat (SSR). **African Journal of Agricultural Research 17**: 794-801.

Navarro CFA, Balois-Morales R, Zurita JOJ, Jiménez VAO, Ramírez IFP, Varela GB and Rosales PUB (2023) Parthenocarpic and stenospermocarpic mango fruits: A review of possible causative factors. **Bio Ciencias 10**: e1480.

Nordlander J, Satra JS, Mattisson H, Udovychenko K, Lushpigan O, Lose L, Naess H, Leino M, Hjalmarsson I and Garkava-Gustavsson L (2022) Genetic diversity in gooseberry (Ribes uva-crispa), as estimated with SSR markers. **Scientia Horticulturae 306**: 111438.

Ravishankar KV, Bommisetty P, Bajpai A, Srivastava N, Mani BH, Vasugi C, Rajan S and Dinesh MR (2015) Genetic diversity and population structure analysis of mango (*Mangifera indica*) cultivars assessed by microsatellite markers. **Trees 29**: 775-783.

Ravishankar KV, Bommisetty P, Lavanya B, Mani BH and Dinesh MR (2017) Development and characterization of microsatellite loci from mango (*Mangifera indica* L.). **Indian Journal of Biotechnology 2**: 250-253.

Ravishankar KV, Mani BH, Anand L and Dinesh MR (2011) Development of new microsatellite markers from mango (*Mangifera indica*) and cross-species amplification. **American Journal of Botany 98**: e96-e99.

Rocha A, Salomão TM, Siqueira DL, Cruz CD and Salomão LDC (2014) Identification of 'Ubá' mango tree zygotic and nucellar seedlings using ISSR markers. **Ceres 5**: 597-604.

Santos SS, Gomes FAL, Silva LS, P Neto VB, Silva MTL, Silva ACR and Cavalcante ÍHL (2024) Rootstock affects phytotechnical attributes, gas exchange, and carbohydrate accumulation in mango scion. **Polish Society for Horticultural Science 36**: 337-350.

Song M, Wang H, Fan Z, Huang H and Ma H (2023) Advances in sequencing and key character analysis of mango (*Mangifera indica* L.). **Horticulture Research 2**: 1-16.

Sridhar D, Ghosh B, Das A and Pramanik K (2022) Assessment of genetic diversity using morphological and molecular markers in traditional cultivars of Mango (*Mangifera indica* L.). **Indian Journal of Traditional Knowledge 2**: 404-413.

Srivastav M, Singh SK, Prakash J, Singh R, Sharma N, Ramchandra S, Devi R, Gupta A, Mahto AK, Jayaswal PK, Singh S and Singh NK (2021) New hyper-variable SSRs for diversity analysis in mango (*Mangifera indica* L.). **India Journal of Genetics and Plant Breeding 1**: 119-126.

Tardivo C, Patel S, Bowman KD and Albrecht U (2025) Nursery characteristics and field performance of nine novel citrus rootstocks under HLB-endemic conditions. **HortScience 60**: 931-939.

Viruel MA, Escribano P, Barbieri M, Ferri M and Hormaza JM (2005) Fingerprinting, embryo type and geographic differentiation in mango (*Mangifera indica* L., Anacardiaceae) with microsatellites. **Molecular Breeding 4**: 383-393.

Yan J, Zheng B, Wang S, Xu W, Qian M, Ma X and Wu H (2024) Genetic diversity and fingerprinting of 231 mango germplasm using genome SSR markers. **International Journal of Molecular Sciences 25**: 1-14.

Yan P, Xie Z, Feng K, Qiu X, Zhang L and Zhang H (2023) Genetic diversity analysis and fingerprint construction of Korean pine (*Pinus koraiensis*) clonal seed orchard. **Frontiers in Plant Science 13**: 1079571.

Zhang X, Chen W, Yang Z, Luo C, Zhang W, Xu F, Ye J and Liao Y (2024) Genetic diversity analysis and DNA fingerprint construction of *Zanthoxylum* species based on SSR and iPBS markers. **Plant Biology 24**: 843.