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RESEARCH

Genetic Diversity of 'Ubá' Mango Tree Using ISSR Markers

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Abstract In this study, the genetic diversity of 'Ubá' mango trees cultivated at the Zona da Mata of Minas Gerais State, Brazil, was assessed, to identify whether there is variability in the plants grown in the region, justifying the mass selection as a breeding method. We used 102 accessions. Leaves were collected for extraction of genomic DNA, which was amplified with nine ISSR primers. The data obtained by the analysis of electrophoretic patterns were arranged in a binary matrix, considering 0 for the absence and 1 for the presence of bands. Based on these data, we performed the analysis of genetic dissimilarity and carried out the cluster analysis by the methods of Tocher and graphical dispersion. The most similar accessions are 144 and 150, both coming from Ubá, while the most divergent ones are 29 and 97, from Visconde do Rio Branco. The grouping by the Tocher method separated the accessions into six groups, 94.1% of which were allocated in the first group and showed that there is no separation of accessions depending on the sampling sites. The 3D scatter plot reinforces this conclusion. There is genetic variability among the accessions of 'Ubá' mango tree evaluated. Therefore, it is possible to make mass selection in open-pollinated populations.

Keywords *Mangifera indica* L. · Genetic variability · Mass selection · Jaccard index · Polyembryony

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Introduction

Mango is one of the most cultivated tropical fruits in the world, both for fresh and industrial consumption. The Zona da Mata of Minas Gerais State, Brazil, stands out as a producer of 'Ubá' mango, a cultivar with excellent conditions for industrialization due to its superior characteristics, such as pulp color and flavor, besides the great appreciation for fresh consumption.

The 'Ubá' mango tree is well adapted to the edaphoclimatic conditions and grows spontaneously in almost all the municipalities of the Zona da Mata. A large proportion of the plants come from seeds (non-grafted seedlings), with wide variety in fruit size, pulp color, susceptibility to anthracnose, production, etc. However, this variation can be only phenotypic, since this cultivar produces polyembryonic seeds containing both the nucellar and zygotic embryos. Nucellar embryos originate plants genetically identical to the parent plant. Therefore, in a genetic breeding program based on mass selection, there would be unnecessary spending of time and financial resources if genetically similar plants selected based on phenotypic characteristics were reproduced and cultivated in the same location and evaluated for several years.

The selection of mother plants must be performed considering the genetic similarity based on the information about DNA molecular markers. These are important breeding tools, since they do not suffer environmental influence. The identification of differences at DNA level may increase the efficiency of the Breeding Program [1]. Thus, in the Genetic Breeding Program of the 'Ubá' mango tree developed at the Federal University of Viçosa since 2004, we will use Inter simple sequence repeat molecular markers (ISSR) for the study of genetic diversity at DNA level, allowing the exclusion of genetically identical material.

ISSR markers are dominant and based on the amplification of regions between DNA adjacent microsatellite sequences via polymerase chain reaction (PCR) and combine most advantages of microsatellites and AFLP, as well as the universality of RAPD markers. They are highly polymorphic and reproducible, do not require previous knowledge about the genome and are relatively inexpensive [2]. They are also versatile and can be used in plant breeding programs, besides evolution studies, due to their ability to show differences among cultivars [3]. They are adequate for studies on genetic diversity, phylogeny, marked or labeled genes, gene mapping, and evolutionary biology [4–6]. The reproducibility presented by ISSR markers is the main difference from RAPD markers, the reproducibility rate of which is low, and polymorphism, high [7].

ISSR markers identified in mango were employed to assess the genetic diversity among 70 cultivars in India [8], and the results showed the efficiency of these for the study of genetic diversity. In Brazil, few studies have been developed with DNA molecular markers in mango trees. RAPD markers have been employed to evaluate the genetic diversity of mango tree genotypes [1, 9, 10], to identify the genetic origin of seedlings of mango tree 'Rosinha' [11], and determine the parents in mango tree hybrids [12]. AFLP markers were employed to analyze the genetic relations of 105 accessions of mango trees of the Embrapa Semiárido germplasm bank [13].

The objective of this study was to assess the genetic diversity of 'Ubá' mango trees cultivated at the Zona da Mata of Minas Gerais State, Brazil, to identify whether there is variability in the plants grown in the region, justifying the mass selection as a breeding method.

Materials and Methods

One hundred and two accessions of 'Ubá' mango trees derived from open pollination from cities of the Zona da Mata of Minas Gerais State, Brazil, were used. Two of them were from Viçosa, identified by the numbers 1 and 101; 48 were from Visconde do Rio Branco, identified by the numbers 2, 5, 10, 12, 13, 14, 15, 16, 18, 19, 20, 24, 25, 26, 28, 29, 35, 36, 38, 39, 41, 43, 44, 49, 51, 53, 55, 58, 59, 60, 62, 64, 68, 71, 72, 75, 76, 77, 79, 81, 82, 84, 85, 87, 93, 97, 98, and 102; and 52 were from Ubá, identified by the numbers 103, 105, 107, 109, 112, 113, 118, 119, 121, 124, 125, 128, 133, 136, 137, 139, 141, 143, 144, 145, 148, 149, 150, 151, 152, 153, 155, 156, 157, 158, 159, 160, 161, 164, 165, 166, 169, 174, 175, 176, 177, 181, 182, 184, 185, 187, 189, 191, 192, 195, 198, and 199. The mango trees were selected in different rural properties, according to the fruit load in the plant, size and aspect of the fruits, and incidence of anthracnose in leaves and fruits.

Mature leaves of each mango tree were selected for genetic characterization. The leaves were identified and maintained in styrofoam boxes with ice to be transported from the collection sites to the Laboratório de Análise de Frutas of the Federal University of Viçosa (UFV), where they were frozen in liquid nitrogen. Then, they were stored in a freezer at −80°C, in the Laboratório de Biologia Molecular of the Departamento de Biologia Geral of the UFV until the time of the extraction of the genomic DNA.

The DNA of the 102 plants was extracted according to [2], with modifications. Two hundred milligrams of leaves were macerated to powder together with liquid nitrogen, in a porcelain mortar. This macerate received 800 µl of the extraction buffer (Cetyl trimethyl ammonium bromide—CTAB 2%, NaCl 1.4 M, Ethylenediamine tetraacetic acid—EDTA 20 mM, Tris–HCl 100 mM, β-mercaptoethanol 0.4%, PVP 1%, and sodium metabisulfite 20 mM), and the mixture was transferred to 1.5 ml microtubes. They were incubated in water bath for 30 min at 65°C and agitated every 10 min. After incubation, deproteinization was performed with the addition of 600 µl of chloroform: isoamyl alcohol at the 24:1 ratio, under agitation for 10 min. After centrifugation at 16,000 *g* for 5 min, the supernatant was transferred to another 1.5 ml microtube, and a new deproteinization was performed. Then, the nucleic acids (DNA and RNA) were precipitated with the addition of 600 µl of cold isopropanol and 150 µl of ammonium acetate 2.5 M and maintained for at least 2 h in a freezer at −20°C. After centrifugation at 16,000 *g* for 30 min, the supernatant was discarded, the pellet was washed twice with ethanol 70% and dried at room temperature, while the nucleic acids were resuspended in 100 µl of TE buffer (Tris–HCl 10 mM, pH 8.0; EDTA 1 mM pH 8.0). The nucleic acid solution was treated with RNAase (10 mg/ml) to eliminate RNA, with the addition of 3 µl in each 100 µl of sample, with incubation at 37°C, for 30 min. The DNA achieved was stored in a freezer at −20°C. Then, the samples were submitted to agarose gel electrophoresis 0.8% containing 0.2 µg/ml of ethidium bromide to quantify and verify the DNA integrity and purity.

Table 1 Primers used and respective sequences, number of bands, pairing temperatures (Ta), and amplification mixture (AM)

Primer	5′-3′ sequence	Number of bands	Ta (°C)	AM[a]
816	CACACACACACACACAT	16	50	A
823	TCTCTCTCTCTCTCTCC	11	53	A
825	ACACACACACACACACT	12	53	B
835[b]	AGAGAGAGAGAGAGAGYC	16	53	B
846[b]	CACACACACACACACART	16	53	B
891[b]	HVHTGTGTGTGTGTGTG	18	40	B
899	CATGGTGTTGGTCATTGTTCCA	16	53	B
D[b]	HVHCACCACCACCACCACT	21	53	B
Terry[b]	GTGGTGGTGGTGRC	17	53	C

[a] According to Table 2, [b]Primers anchored in the extremity 5′ or 3′; Y = (C or T), R = (A or G), H = (A, C or T), and V = (A, C or G)

Ninety-six primers were tested for the DNA amplification. Ninety-three belonged to the Set 9# of the UBC (University of British Columbia), one (Terry) was used by [2], and the other two (C and D) were designed for bees in the Laboratório de Biofísica e Biologia Molecular by Dr. Tânia Maria Fernandes Salomão. Based on the amplification standard, 19 primers were selected, for which the pairing temperatures and the PCR reactions were optimized. Nine of these primers were chosen, considering the number and resolution of bands, with the exclusion of primers with few bands and weak and unspecific bands. The primer sequence and amplification conditions are found in Table 1.

According to the primer used, the reaction mixture for amplification presented different reagent concentrations, originating three different reaction mixtures for amplification called A, B, and C (Table 1), adjusted according to [2, 3]. The reagents used in each reaction are described in Table 2.

The DNA amplification reaction was processed in PTC-100 thermocycler (MJ Research Inc.) under the following conditions: 3 min at 94 °C (initial denaturation), followed by 40 cycles of 1 min at 92°C (denaturation), 2 min at pairing temperature (variable according to the primer in use, see Table 1), 2 min at 72°C (extension), and one step of final extension of 7 min at 72°C.

Table 2 Reagents and quantities employed for each amplification mixture

Reagent	Quantities (µl) per reaction		
	A	B	C
Buffer 10X[a]	2.5	2.0	2.0
Deoxy-nucleotides (dNTP) 100 µM	1.0	2.5	2.5
DNA polimerase[b] 5U/µl	0.2	0.2	0.2
Primer 5 µM	2.0	5.0	2.0
Genomic DNA[c] 17 ηg/µl	3.0	3.0	3.0
Ultrapure water[d]	11.3	12.3	15.3
Final volume	20	25	25

[a] Buffer composition (500 mM KCl, 100 mM Tris–HCl pH 8,4, 1% Triton X-100, 15 mM $MgCl_2$); [b]Taq DNA Polymerase (Phoneutria—IB buffer)—1U; [c]Approximately 50 ng of genomic DNA; and [d] ultrapure water to complete the reaction volume

The amplification products were separated by agarose gel electrophoresis 1.5%, visualized by staining with ethidium bromide (0.2 µg/ml), and photographed using the AlphaDigiDoc system.

The ISSR fragments were codified with binary characters, determining the presence or the absence of bands. Only legible and unambiguous bands were considered in the counting.

The data were disposed in binary matrix of the presence (1) and the absence (0) of amplified fragments. Based on this matrix, the genetic dissimilarity among the accesses studied was estimated, using the complement of the Jaccard index ($d_{ii'} = 1 - S_{ii'}$) to calculate the genetic distance matrix. The matrix was used as a base for the grouping analysis by the Tocher method and graphic projection of distances in tridimensional space (3D). The data were transformed for the squared distance for graphic projection. All the analyses were carried out with the use of the GENES software system [14].

Results and Discussion

The largest genetic distance, calculated by the complement of the Jaccard index among the 102 accessions evaluated, was found between the accessions 29 and 97 ($d_{ii'} = 0.66$) from the city of Visconde do Rio Branco, while the shortest distance was found between the accessions 144 and 150 ($d_{ii'} = 0.07$), both from Ubá. Therefore, accessions with 100% of similarity were not found, which indicates the absence of duplicates. Reference [1], studying genetic diversity among 25 accessions of 'Rosa' mango tree using RAPD markers, concluded that it is possible to breed this polyembryonic cultivar, the largest genetic distance of which calculated by the Jaccard coefficient was 0.45. In 'Kensington Pride' mango, a polyembryonic cultivar presenting phenotypic variations (irregular production and variation in the type of fruit and susceptibility to pathogens), it was

observed, with RAPD markers, that 15 out of the 27 plants evaluated did not present any different amplified DNA fragments in 10 primers used. In other words, they are clones, and the highest dissimilarity found by the simple index of coincidence was 0.05, and this would be the source of genetic variability for breeding inside this cultivar [15]. Higher diversity was detected among the 102 accessions analyzed in this study. Therefore, it is possible to select accession of 'Ubá' mango tree adapted to the conditions of soil and climate of the cultivation region.

The genetic similarity among mango tree cultivars is relatively high. Reference [3], using ISSR markers to evaluate genetic variation among 22 cultivars of mango tree cultivated in Thailand, found, by the Nei and Li's coefficient, the lowest similarity of 0.516 ('Neelum' and 'Tommy Atkins') and the highest of 0.831, between 'Brooks' and 'Edward' [16], using RAPD markers to analyze 25 mango cultivars of the germplasm collection (National Clonal Germoplasm Repository, Miami), observed, by the simple matching coefficient, the lowest similarity of 0.56 and the highest similarity of 0.88. Reference [17] achieved, by the Jaccard coefficient, the lowest similarity of 0.67 and the highest of 0.92 for 36 mango cultivars from a germplasm collection in China (Guangxi Academy of Agricultural Sciences), with cpISSR markers. Therefore, it is observed that, among the accessions of 'Ubá' mango tree analyzed, there are accessions with higher dissimilarity, compared with the cultivars studied by the authors previously mentioned, which demonstrates that it is possible to select plants with superior characteristics by the mass selection method adopted by the 'Ubá' Mango Tree Genetic Breeding Program developed by the UFV.

Although 'Ubá' mango trees are polyembryonic, with the ability to generate plants genetically identical to the parent plant, due to their nucelar embryos [18], no duplicates (clones) were found among the 102 accessions. In other words, no accessions with 100% of similarity were found. The accessions, 144 and 150, which presented higher similarity (93%) and other accessions with similarity higher than 90% were not regarded as duplicates, although they were genetically very close. Mango tree cultivars have been differentiated with indices of similarity of 97%, as observed between the cultivars 'Manila' and 'Carabao', using AFLP markers [13], and similarity of 93% between these two cultivars verified using SSR markers [19]. The cultivars 'Totapuri' and 'PKM-1' were considered different, with 95% of similarity [20].

The absence of duplicates among the accessions studied explains the collection of scions to the formation of grafted trees and their cultivation in the same location, to evaluate the phenotypic characters for several years and eventually select accessions with superior characteristics. Even small, the genetic differences among the sampled materials may affect some phenotypic character that is useful for the culture, as observed by [9]. These authors, using RAPD markers to evaluate the genetic relations among accessions of 'Keitt Pequena', 'Keitt' and 'Tommy Atkins', found the similarity index of 0.927 between the accesses of 'Keitt Pequena' and 'Keitt'. Based on this similarity index, they inferred that the differences in the size of plants and fruits, observed between the cultivars 'Keitt Pequena' and 'Keitt' may be genetic.

The grouping by the Tocher method allowed the separation of accessions into six groups (Table 3), with 94.1% of the accessions allocated in the group 1, which was subdivided into 16 subgroups. The six groups formed do not present separation from the accessions related to the sampling locations, which indicates low variability.

The presence of 94.1% of the accessions in the same group may be explained by the prevalence of this type of mango tree in the Zona da Mata region of Minas Gerais and by the fact that this cultivar has polyembryonic seeds, which may be favoring pollination between genetically similar plants, thus reducing genetic variability.

Table 3 Groups formed by the grouping method of Tocher for 102 accessions of 'Ubá' mango tree from Viçosa, Visconde do Rio Branco and Ubá, in Minas Gerais State, Brazil

Groups	Subgroups	Accessions
1	A	144, 150, 149, 156, 155, 141, 158, 161, 136, 181, 153, 118, 176, 18, 148, 151, 152, 177, 160, 112, 43, 38, 137, 187, 165, 166, 124, 75, 60, 41, 164, 139, 81, 125, 59, 16, 55, 39, 105, 19, 20, 174, 159, 107, 184, 199, 72, 1, 62, 87, 189, 14, 13, 77, 44, 51, 53, 157, 102, 71, 103, 182, 28, 12, 58, 2, 36, 25, 185, 76, 119, 10, 5, 175, 128, 15
	B	109, 195
	C	49, 169, 143
	D	98, 191
	E	82, 85
	F	26
	G	192
	H	79
	I	93
	J	198
	L	64
	M	84
	N	101
	O	35
	P	145
	Q	133
2		97, 113
3		24
4		121
5		68
6		29

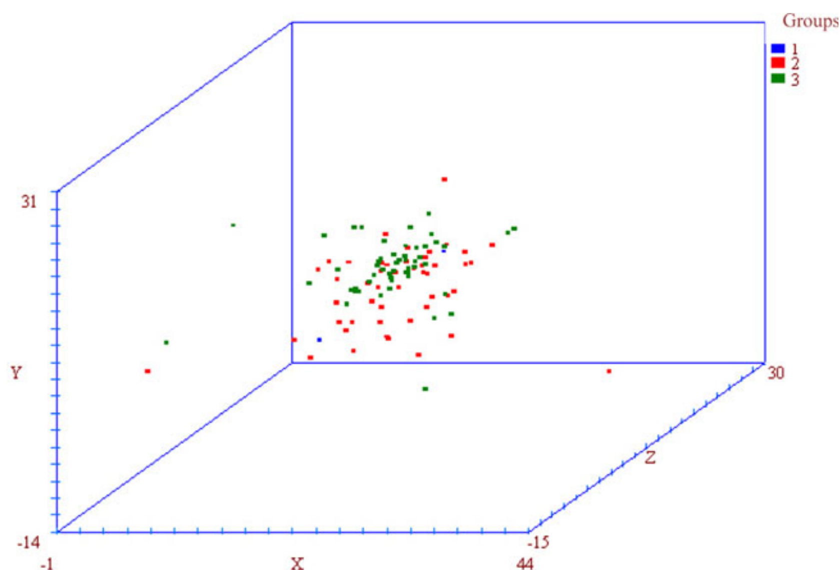

Fig. 1 3D dispersion projection with the data transformed for the squared distance, for the 102 accessions analyzed. *Group 1* accessions selected in Viçosa; *Group 2* Visconde do Rio Branco; and *Group 3* Ubá, Minas Gerais State. Brazil

The dispersion of the measurements of dissimilarity in the tridimensional space (3D) with the data transformed for the squared distance presented distortion coefficient of 24.01%, correlation between the original and estimated distances equal to 0.7423 and stress coefficient of 39.29%. The distortion and stress data were superior to the value considered satisfactory (lower than 20%).

The 3D dispersion graph (Fig. 1) reinforces the lack of group formation per sampling sites, indicating that the existing variability is distributed among the accessions. The same thing is observed in the dendrogram generated by UPGMA (data not shown) and, apparently, the accessions selected in Visconde do Rio Branco present higher variability than those from Ubá.

The absence of accession grouping per sampling sites may be due to the proximity between the sampled cities. The material collection was carried out at the maximum distance between the plants of approximately 49 km, which facilitates material interchange among the farmers of the region. There are climatic differences among Viçosa, Visconde do Rio Branco, and Ubá. Viçosa presents average annual temperature close to 19°C and Visconde do Rio Branco, 21.9°C, while Ubá is considered the warmest city in the Zona da Mata of Minas Gerais, with average annual temperature of 23.5°C. However, these variations do not seem to affect the differences among the accessions of ‘Ubá’ mango tree.

Conclusions

There is genetic diversity among the accessions of the ‘Ubá’ mango trees analyzed, which allows the genetic breeding of this cultivar by means of mass selection.

There was no formation of accession groups according to the sampled locations.

The accessions identified as 29 and 97, from Visconde do Rio Branco, presented the highest genetic dissimilarity.

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References

1. Souza, V. A. B., & Lima, P. S. C. (2004). Genetic variability in mango genotypes detected by RAPD markers. *Acta Horticulturae, 645*, 303–310.
2. González, A., Coulson, M., & Brettell, R. (2002). Development of DNA markers (ISSRs) in mango. *Acta Horticulturae, 575*, 139–143.
3. Eiadthong, W., Yonemori, K., Sugiura, A., Utsunomiya, N., & Subhadrabandhu, S. (1999). Identification of mango cultivars of Thailand and evaluation of their genetic variation using the amplified fragments by simple sequence repeat-(SSR-) anchored primers. *Scientia Horticulturae, 82*, 57–66.
4. Reddy, M. P., Sarla, N., & Siddiq, E. A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application plant breeding. *Euphytica, 128*, 9–17.
5. Ye, C., Yu, Z., Kong, F., Wu, S., & Wang, B. (2005). R-ISSR as a new tool for genomic fingerprinting, mapping, and gene tagging. *Plant Mol Biol Rep, 23*, 167–177.
6. Faleiro, F. G. (2007) Marcadores genético-moleculares aplicados a programas de conservação e uso de recursos genéticos. Planaltina (DF), Embrapa Cerrados, p. 102.
7. Nagaoka, T., & Ogihara, Y. (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theoretical and Applied Genetics, 94*, 597–602.

8. Pandit, S. S., Mitra, S., Giri, A. P., Pujari, K. H., Patil, B. P., Jambhale, N. D., et al. (2007). Genetic diversity analysis of mango cultivars using inter simple sequence repeat markers. *Current Sci, 93*, 1135–1141.
9. Faleiro, F. G., Pinto, A. C. Q., Rossetto, C. J., Fraga, L. M. S., Andrade, S. E. M., & Bellon, G. (2004) Avaliação da origem de variações fenotípicas da manga 'Keitt' cultivada em São Paulo com base em marcadores RAPD. In: Congresso Brasileiro De Fruticultura, 18, Florianópolis. Anais… Florianópolis: SBF, 2004. 1 CD-ROM.
10. Souza, I. G. de B., Diniz, F. M., Souza, V. A. B., Valente, S. E. dos S., Barros, F. B., & Lima, P. S. da C. (2008) Similaridade genética entre genótipos de manga com base em marcadores RAPD. In: Costa, N.A. and Costa, A.F.S. (Orgs.). Congresso Brasileiro De Fruticultura, 20, 2008, Vitória. Anais… Vitória: Incaper,. 1 CD-ROM.
11. Cordeiro, M. C. R., Pinto, A. C. Q., Ramos, V. H. V., Faleiro, F. G., & Fraga, L. M. S. (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.
12. Cordeiro, M. C. R., Pinto, A. C. Q., Ramos, V. H. V., Faleiro, F. G., & Fraga, L. M. S. (2006). RAPD markers utilization and other parameters in the determination of mango hybrids genitors. *Revista Brasileira de Fruticultura, 28*, 164–167.
13. Santos, C. A. F., Neto, F. P. L., Rodrigues, M. A., & Costa, J. G. (2008). Similaridade genética de acessos de mangueira de diferentes origens geográficas avaliadas por marcadores AFLP. *Revista Brasileira de Fruticultura, 30*, 736–740.
14. Cruz, C. D. (2008) Programa GENES—Diversidade genética. Viçosa (MG), UFV, p. 278.
15. Bally, I. S. E., Graham, G. C., & Henry, R. J. (1996). Genetic diversity of Kensington mango in Australia. *Australian Journal of Experimental Agriculture, 36*, 243–247.
16. Schnell, R. J., Ronning, C. M., & Knight, R. J. (1995). Identification of cultivars and validation of genetic relationships in *Mangifera indica* L. using RAPD markers. *Theoretical and Applied Genetics, 90*, 269–274.
17. Xin-Hua, H. E., Yong-Ze, G. U. O., Yang-Mil, L. I., & Sh-Jin, O. U. (2007). Assessment of the genetic relationship and diversity of mango and its relatives by cpISSR marker. *Agricultural Sciences in China, 6*, 137–142.
18. Strurrock, T. T. (1967). Nucellar embryos of the mango. *Proceedings of the Florida State Horticultural Society, 80*, 350–354.
19. Viruel, M. A., Escibano, P., Barboero, M., Ferri, M., & Hormaza, J. I. (2005). Fingerprinting embryo type and geographic differentiation in mango (*Mangifera indica* L., Anacardiaceae) with microsatellites. *Molecular Breeding, 15*, 383–393.
20. Hemanth-Kumar, N. V., Narayanaswamy, P., Theertha Prasasd, D., Mukunda, G. K., & Sondur, S. N. (2001). Estimation of genetic diversity of commercial mango (*Mangifera indica* L.) cultivars using RAPD markers. *Journal of Horticultural Science and Biotechnology, 76*, 529–533.