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Morpho-Molecular Diversity of Cashew Nut (*Anacardium occidentale* L.) Germplasm of Bangladesh

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Abstract

The present investigation was conducted to identify the genetic variability in morphological characteristics among 12 cashew nut germplasm of Bangladesh. The morphological parameters such as nut length, width, single nut weight, and 100-nut weight varied significantly ($p \leq 0.01$) among the germplasm studied. The shape and color of the cashew nuts and cashew apples were also variable. The RAPD analysis of the germplasm showed 71.43% polymorphism on average. The cluster analysis results revealed two distinct groups; cluster-I was made up of two genotypes, GP-7 and GP-8, while cluster-II consisted of ten genotypes. The maximum genetic distance (0.50) was exhibited between GP-4 and GP-8, while the minimum (0.107) was between GP-1 and GP-2, and between GP-2 and GP-3. The results of nut morphological characteristics and genetic relationships in cashew nut germplasm may be utilized in describing of new varieties and for plant improvement programs.

Introduction

Cashew (*Anacardium occidentale* L.) is a valuable fruit tree, a member of the Anacardiaceae family (Rao and Swamy 1994). It is an evergreen perennial tree that has thick foliage and can reach heights to 15 meters (Nakasone and Paull 1998). The cashew is a native plant of tropical America. Major cashew-growing countries are India, Brazil, Tanzania, Mozambique, Kenya, and Vietnam (Dasmohapatra et al. 2014).

Most of the Indian states that are located near the Bay of Bengal are well known for cashew farming. Bangladesh is also situated in the same coastal belts and has a favorable agro-climate, but the potentiality for commercial cashew cultivation is still unexplored;

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although about a half-century back cashew was introduced in Bangladesh in its eastern hilly areas (DAE 2021). The demand for cashew kernels and their by-products is rising on the global market. Commercial cashew cultivation in the coastal plain of Bangladesh might be a good source of employment and foreign currency income. To satisfy the market's needs, it is crucial and necessary to choose and develop high-performing varieties. The agro-morphological and genetic characteristics of cashews grown locally are now little known (Trapaga et al. 2020). Therefore, phenotypic characterization is essential for the purpose of determining phenotypically superior plants (Hawerroth et al. 2019). It is also highly desired to use molecular markers to aid in pre-breeding and breeding processes in order to speed up the varietal development.

DNA markers are well known to be extremely effective for assessing the diversity of plant species (Yildiz et al. 2021). RAPD marker, produced by PCR using random primers, is the most accessible and affordable method for detecting DNA polymorphism, enabling the identification of genetic links in plants (Nagori et al. 2018). The RAPD molecular tool can be used to characterize cashew accessions for morphological features, analyze genetic relationships, and describe new varieties (Samal et al. 2003). In the present investigation, nut morphological diversity and genetic diversity of twelve cashew germplasm were analyzed with the objectives of characterizing nuts of different cashew germplasm collected from Bangladesh and investigating the variability of cashew germplasm using the RAPD marker.

Materials and Methods

This study was carried out at Plant Breeding and Biotechnology Laboratory, Agrotechnology Discipline of Khulna University, Bangladesh. Fresh cashew nuts with apples of 12 germplasm were collected from Chittagong, Bandarban, Mymensingh, and Khulna districts of Bangladesh. The age of the mother trees varies from 5-20 years, and their origin is mostly ambiguous. Immediately after collection, various qualitative data e.g., nut color and shape; apple color and shape, and quantitative data e.g., nut length, width, and weight were recorded. The experiment was conducted laid out in a completely randomized design (CRD) with three replications per germplasm. Fifteen nuts along with apples were considered as a replication.

Sterilized tender leaves of cashew seedlings were lipolyzed and ground to powder in liquid nitrogen for DNA extraction through the DNAzol protocol (Chomczynski et al. 1997 & 1998). The DNA samples were quantified using a UV spectrophotometer at 260 nm and the final concentrations were adjusted to 1.00 ng/ml for PCR. The PCR reaction was carried out in a final volume of 20 µl reaction mixture containing 10 mM dNTPs (0.2 µl), 5 U/µl taq polymerase (0.2 µl), 100 pM/µl primers (1.0 µl), 10× reaction buffer without $MgCl_2$ (1.0 µl), 25 mM $MgCl_2$ (1.2 µl), 30 ng μL^{-1} DNA template (1.0 µl) and Molecular water (Sigma Aldrich, USA) (15.4 µl). This reaction mixture was involved in PCR amplification conditions (denaturation at 94°C for 1 min, annealing at 35°C for 1min,

extension at 72°C for 2 mins, 35 cycles, final extension at 72°C for 10 mins, holding at 4 °C for 10 mins and end). Amplified PCR products were electrophoresed in agarose gel (1%) in TAE buffer and visualized after staining with ethidium bromide. A study of the genetic diversity of each twelve germplasm was scored manually for the presence or absence of a particular amplification product. A molecular weight marker of 1 kb (Direct load, Sigma Aldrich, USA) was used.

Table 1. List of random primers with GC content and Tm of primers.

N/N	Primer Code	Sequence (5'–3')	GC Content (%)	Tm of Primer (°C)
1	OPE-02	GGT GCG GGA A	70.00%	30
2	OPA-11	CAA TCG CCG T	60.00%	30
3	OPA-12	TCG GCG ATA G	60.00%	30
4	OPA-13	CAG CAC CCA C	70.00%	30
5	OPA-14	TCT GTG CTG G	60.00%	30
6	OPE-18	TGC GGC TGA G	70.00%	30
7	OPA-20	GTT GCG ATC C	60.00%	30

Variations among the cashew nuts based on morphological characters were analyzed for ANOVA and the correlation of different parameters was assessed by using STAR (Statistical Tool for Agricultural Research, version 2, IRRI, Los Banos, Philippines). PCR amplifications resolved on agarose gel were scored to generate binary data by giving scores of 1 and 0 for the presence and absence of amplified bands, respectively at different levels of molecular weight, for all the germplasm. Binary data were generated for all the primers and subjected to the PAUP 4.0 software program for interpretation of the results. The dissimilarity matrix of twelve germplasm was generated based on band sharing data and a weighted neighbor-joining tree was constructed.

Results and Discussion

Data of different qualitative and quantitative traits on nut and apple morphology of 12 collected cashew germplasm of Bangladesh are presented in Tables 2-5 and Figs 1-2. It was evident that all the cashew germplasm exhibited distinguishing nut and apple morphological traits and differed from each other significantly.

Kidney-shaped nut was found prominent (66.67%) in GP 1, 2, 3, 4, 5, 10, 11, and 12 followed by oblong-ellipsoid (33.33%) in GP 6, 7, 8, and 9 (Fig. 1 and Table 2). Four nut color was found among the germplasm (Fig 1) where brown color was maximum in eight germplasm (GP 4, 5, 7, 8, 9, 10, 11, 12) followed by ash gray in two (GP-2 and GP-3), whitish-gray in one (GP-1), and blackish gray in one (GP-6).

Table 2. Qualitative traits of nut and apple of collected cashew germplasms.

Germplasm number	Collection location	Cashew nut		Cashew apple	
		Shape	Color	Shape	Color
GP-1	K U	Kidney	Whitish gray	Cylindrical	Yellow
GP-2	K U	Kidney	Ash gray	Cylindrical	Yellow
GP-3	K U	Kidney	Ash gray	Round	Yellow
GP-4	A B	Kidney	Brown	Conical	Yellow
GP-5	A B	Kidney	Brown	Conical	Reddish yellow
GP-6	H C	Oblong-ellipsoid	Blackish gray	Round	Red
GP-7	H C	Oblong-ellipsoid	Brown	Cylindrical	Red
GP-8	H C	Oblong-ellipsoid	Brown	Cylindrical	Red
GP-9	BAU	Oblong-ellipsoid	Brown	Round	Yellow
GP-10	B B	Kidney	Brown	Conical	Light yellow
GP-11	K U	Kidney	Brown	Conical	Yellow
GP-12	K U	Kidney	Brown	Cylindrical	Red

*GP = Germplasm, KU = Khulna University, A B = Aziznagar, Bandarban, HC = Hathazari, Chottogram, BAU = Bangladesh Agricultural University, B B = Balaghata, Bandarban.

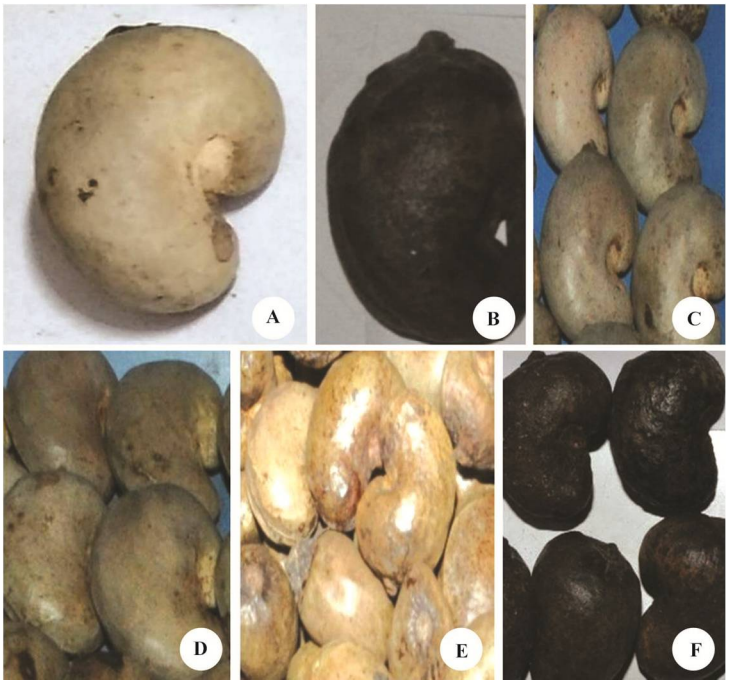

Fig. 1. Nut characteristics of collected cashew germplasm (Shape: A. kidney, B. Oblong-ellipsoid, and Color: C. Whitish gray, D. Ash gray, E. Brown, F. Blackish gray).

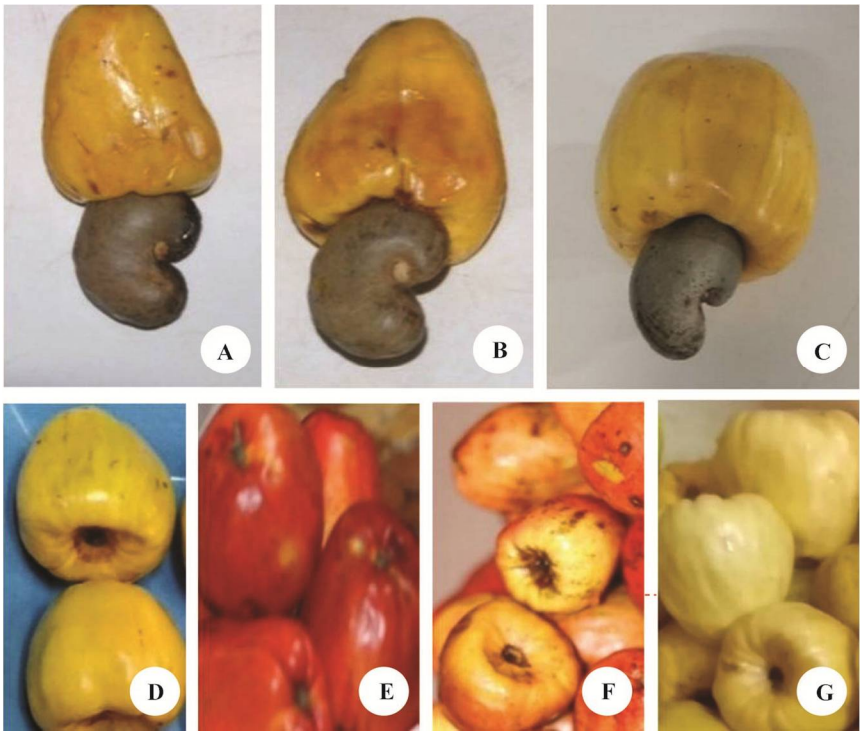

Fig. 2. Apple characteristics of collected cashew germplasm (Shape: A. Cylindrical, B. Conical, C. Round and Color: D. Yellow, E. Red, F. Radish yellow, G. Light yellow).

The maximum apple shape was cylindrical (GP-1, 2, 7, 8, 12) followed by conical (GP-4, 5, 10, 11), and round (GP-3, 6, 9). The yellow apple color was prominent and found in six germplasm (GP 1, 2, 3, 4, 9, 11) followed by red in four (GP 6, 7, 8, 12), radish-yellow in one (GP 5), and light-yellow in one (GP10). Ona et al. (2017) found the same yellow to red apple color in eight cashew nut germplasm of Bangladesh. Dorajeerao et al. (2002) found yellow and yellow-red apple colors among 14 clones of cashew. Mangal (2016) found yellow in ten and red in four genotypes (Table 2).

Assessment of agro-morphological variations, including qualitative and quantitative traits is commonly used to estimate crop genetic diversity (Sunita et al. 2021; Sousa et al. 2019; Tarpaga et al. 2020). Such traits are important in the breeding process, as they are easy to evaluate (Ngure et al. 2021).

The results from ANOVA (Table 3) showed that the cashew germplasm were varied significantly ($p \leq 0.01$) in respect of nut length, nut width, single nut weight, and 100-nut weight. The mean squares are the values used in the subsequent test for significant differences between the group means. As variability is an essential tool for crop improvement (Cobb et al. 2019), the genetic improvement of the cashew nut can be undertaken from these local germplasm, relying on phenotypic selection.

Table 3. Analysis of variance (ANOVA) for nut characteristics of cashew germplasm.

Source of variation	DF	Nut length (mm)	Nut width (mm)	Single nut weight (g)	Hundred nut weight (g)
Replication	4	9.12	1.86	0.46	4619.02
Germplasm	11	23.08**	21.86**	4.11**	41120.34**
Error	44	2.79	3.63	0.50	5019.96
CV%		5.67	10.90	13.25	13.25
SD		2.15	2.09	0.91	90.68

**Significant at 1% level.

Table 4. Nut characteristics of collected cashew germplasm.

Germplasm number	Nut length (mm)	Nut width (mm)	Single nut weight (g)	Hundred nut weight (g)
GP-1	26.89 d	14.56 d	3.77 f	377.40 f
GP-2	27.23 d	15.34 cd	4.21 ef	421.40 ef
GP-3	28.97 cd	15.44 cd	5.22 a-f	521.60 a-f
GP-4	28.84 cd	17.66 a-d	5.54 a-e	554.40 a-e
GP-5	28.16 cd	17.96 a-d	6.18 a-d	617.80 a-d
GP-6	33.55 a	19.32 a-c	6.58 a	658.20 a
GP-7	29.56 b-d	17.46 a-d	5.00 b-f	500.00 b-f
GP-8	30.48 a-d	15.29 cd	4.68 d-f	467.80 d-f
GP-9	28.15 cd	16.87 b-d	5.25 a-f	525.00 a-f
GP-10	27.87 cd	18.27 a-d	4.92 c-f	492.20 c-f
GP-11	32.93 ab	21.06 a	6.29 a-c	629.40 a-c
GP-12	31.15 a-c	20.40 ab	6.51 ab	651.40 ab
LSD (0.05)	3.6445	4.1548	1.5456	154.5559
LS	**	**	**	**

Data in a column followed by same letters are not different significantly according to LSD test.
LS= Level of significance.

There was a significant ($p \leq 0.01$) variation in nut length of the studied cashew germplasm and it was the highest in GP-6 (33.55mm) and the lowest in GP-1 (26.89). The nut width of 12 cashew germplasm was also varied significantly ($p \leq 0.01$), it was the highest (21.06 mm) in GP-11 and the lowest (14.56 mm) in GP-1. Single nut weight and hundred nut weight also had significant differences ($p \leq 0.01$) and it was the highest (6.58 g and 658.20 g, respectively) in GP-6 and the lowest (3.77 g and 377.40 g, respectively) in GP-1. So according to the above results of nut characteristics among 12 germplasm, GP-6

was the best (Table 4). Kehinde et al. (2015) found that nut fraction and biotype had a considerable effect on nut length, nut width, and 100-nut weight.

Table 5. Correlation matrix of the morphological characters of cashew germplasm.

	NL	NW	SNW	100NW	NS	NC	AC	AS
NL								
NW	0.55							
SNW	0.33	0.86**						
100NW	0.33	0.86**	0.99**					
NS	0.32	-0.07	0.06	0.06				
NC	0.44	- 0.10	-0.16	-0.16	0.29			
AC	-0.39	-0.19	-0.38	-0.38	-0.59*	-0.27		
AS	-0.33	0.26	0.48	0.48	-0.14	-0.18	0.14	
MPH	0.30	0.31	0.26	0.26	0.09	0.05	0.11	0.08

NL= Nut length, NW= Nut width, SNW= Single nut weight, 100NW= Hundred nut weight, NS= Nut shape, NC= Nut color, AC= Apple color, AS= Apple shape, MPH= Mother plant height.

A high positive correlation (0.9996) was observed between single nut weight (SSW) and 100-nut weight (100NW), followed by nut width (NW) and single nut weight (SNW). Apple color (AC) had a negative significant correlation with nut shape but positive correlation with apple shape and plant height (Table 5).

Table 6. Primers used for RAPD analysis and number of amplified DNA fragments.

Sl. No.	Primer code	Maximum number of amplified bands	Number of Polymorphic bands	Number of Monomorphic bands	Polymorphism (%)
1	OPE-02	01	0.0	01	0.0
2	OPA-11	05	01	04	20
3	OPA-12	05	03	02	60
4	OPA-13	05	05	0.0	100
5	OPA-14	03	03	0.0	100
6	OPE-18	05	05	0.0	100
7	OPA-20	04	03	01	75
	Total	28	20	08	71.43

The RAPD technique had been successfully used in a variety of taxonomic and genetic diversity studies (Rodriguez et al. 1999: Alam et al. 2009). Genetic variation was detected among 12 cashew germplasm by using RAPD markers. Seven random primers

viz. OPE-02, OPA-11, OPA-12, OPA-13, OPA-14, OPE-18, and OPA-20 were used to amplify DNA segments. Among the amplified DNA products two types of bands were found, *viz.* monomorphic and polymorphic bands. Monomorphic bands are those which are present in all individuals, polymorphic were present in one or more, but not all individuals, and unique ones were present in at least one individual not in any other (Mehetre et al. 2004).

In the current study, a total of 28 amplified DNA fragments were obtained using seven primers (Table 6). Primer OPE-02 amplified the fewest number of bands and all were monomorphic, whereas primers OPA-11, OPA-12, OPA-13, and OPE-18 produced the most amplified fragments. Overall, 71.43% polymorphism was observed, which indicates the genetic diversity of the twelve cashew germplasm studied (Table 6). Three of the seven primers generated 100% polymorphism, whereas the remaining three gave 75%, 60%, and 20% of it, respectively. So, on average each primer produced 10.20% polymorphism. This is explicable as the product amplification depends upon the sequence of random primers and their compatibility with genomic DNA. Sources of polymorphism in RAPD assay may be due to deletion, addition, or substitution of the base within the priming site sequence (Willams et al. 1990). Bhadra et al. (2019) also found similar results i.e., 74.12% polymorphism on average with four RAPD markers among six cashew germplasm. Dasmohapatra et al. (2014) reported 81.55% to 89.55% polymorphism by observing 25 cashew genotypes following the RAPD technique. Samal et al. (2003) also reported genotypic-dependent polymorphism in cashew.

Out of seven primers, only OPE-02 produced monomorphic band, and these amplified DNA fragments ranged from 0. 01 to 0.05 kb (Fig. 3A). Primer OPE-18 (Fig. 3E) and OPA-13 produced DNA fragments of 0.01 to 0.4 kb size and amplified five polymorphic bands (Table 6). Primer OPA-14 (Fig. 3D) produced bands of 0.02 to 0.25 kb size and amplified three polymorphic bands.

The primer OPA-11 (Fig. 3B) amplified DNA fragments with variable sizes from 0.01 to 0.2 kb, OPA-12 (Fig. 3C) produced bands of 0.02 to 0.25 kb size, and OPA-20 (Fig. 3F) produced bands with the range from 0.02 to 0.80 kb, and amplified both monomorphic and polymorphic bands. Here, primers OPA-13 and OPE-18 generated greater numbers of amplified DNA fragments and showed distinct polymorphism. The PCR amplified products using RAPD primers are shown in Fig. 3 (A-F).

These findings demonstrated the applicability of random PCR primers for the study of characterization and evaluation of intra-specific polymorphisms among the germplasm of cashew. The DNA bands resulting from each primer may be different in both size and amount of DNA bands. Chavan et al (2013) found similar results using 30 RAPD markers in 11 cashew genotypes, out of 22 bands, eight bands were monomorphic and 14 were polymorphic which provided 63.33 % polymorphism.

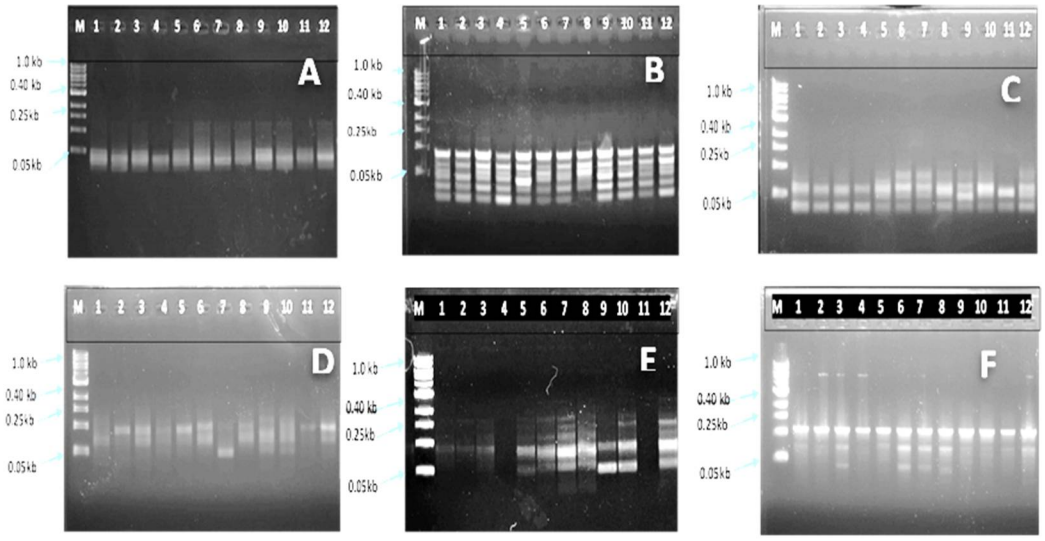

Fig. 3. Gel image of amplified DNA fragments using primers (Primer: A. OPE-02, B. OPA-11, C. OPA-12, D. OPA -14, E. OPE -18, F. OPE -20). Number 1 to 12 indicates germplasm and "M" indicates the Ladder (1Kb).

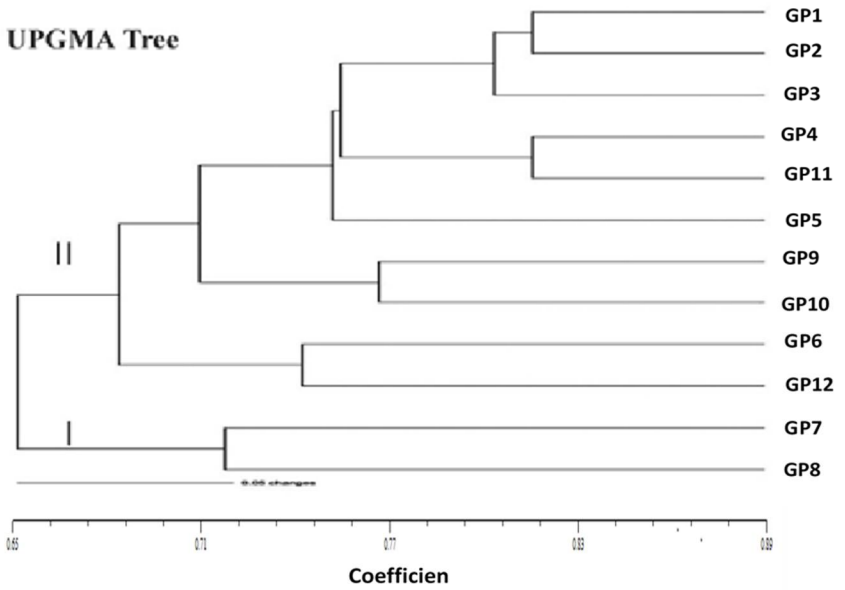

Fig. 4. Dendrogram generated from polymorphic data of seven primers through RAPD analysis of 12 cashews.

Two major clusters were found with (UPGMA) cluster analysis i.e. major cluster-I and major cluster-II from the cluster analysis of twelve selected cashew germplasms (Fig. 4). Cluster-II consisted of 10 cashew germplasm that originated from different locations

in Bangladesh but Cluster-I contained only two germplasms GP-7 and GP-8 collected from the same location, Hathazari Horticulture Center, Chottogram. The maximum genetic distance between the groups was 50% which indicates a wide range of genetic variation within this species. Dhanaraj et al. (2002) estimated the diversity among 90 cashew germplasm using RAPD markers and from the dendrogram, it was confirmed that the diversity of Indian cashew collections can be considered to be moderate'' to ``high''. Hepsibha et al. (2010) also found wide variation within six accessions of *Azima tetracantha* (Lam) using RAPD markers with average (UPGMA) cluster analysis.

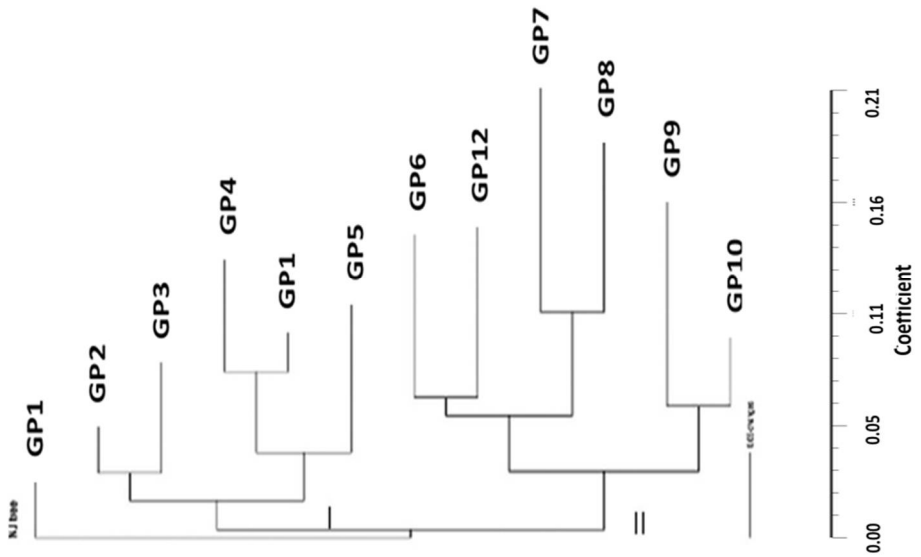

Fig .5. Neighbor-joining tree of twelve cashew germplasm.

The neighbor Joining (NJ) tree demonstrates the evolutionary relationship among the cashew nut germplasm. A dendrogram was constructed using the neighbor-joining method of cluster analysis separated all the 12 germplasm into two clusters where GP-1 (Fig 5) was the common emerging point (ancestor) with the highest inter-varietal similarity index (89.3%). Cluster I contain five germplasm and the rest of the germplasm belongs to cluster II. These five germplasm were collected from Khulna University (GP-2, GP-3, GP-11), and Aziznagar Bandarban (GP-4, GP-5) (Table 4). From the analysis, it was found that the germplasm of cluster I were very close to GP-1 than the germplasm of cluster II. The genetic structure of plant populations reflects the interactions of many different processes such as the long-term evolutionary history of the species (e.g., shifts in distribution, habitat fragmentation, and/or population isolation), mutation, genetic drift, mating system, gene flow, and selection (Slatkin 1987). All of these factors can lead to complex genetic structuring within populations. So, genetic diversity has great importance to the sustainability of plant populations (Wang et al. 2007).

Analyzing the matrix constructed with the amplification data, it was observed a coefficient of dissimilarity between the pair's maximum distance was 50% between GP-4 and GP-8; which were collected from Aziznagar Bandarban and Hathazari Chottogram. The minimum pair-wise distance was found 10.7% between three pairs i.e GP-1 and GP-2, GP-2 and GP-3 collected from Khulna, and GP-4 and GP-11, collected from Aziznagar Bandarban and Khulna University, respectively. The lowest genetic distance (0.107) represented that germplasm pairs were very close to each other with the highest inter-varietal similarity index (89.3%). High similarity indices suggest that the individuals in the population have close genetic relations among them. This situation can arise in natural populations when there is a possibility of free/random pollen flow and fertilization. The genetic similarity of the samples slightly correlated with their close geographic locations (Sayed et al. 2009). The range of genetic distance of 12 cashew germplasm were 0.107 to 0.500 which indicated the presence of genetic variability among the 12 cashew germplasm (Table 7). High diversity is the reflection of adaptation to the environment, which is beneficial to its propagation, resource conservation, the domestication of wild species, and the screen of specified locus.

Table 7. Genetic diversity index from RAPD data of 12 cashew germplasms.

	GP2	GP3	GP4	GP5	GP6	GP7	GP8	GP9	GP10	GP11	GP12
GP1	0.107	0.143	0.214	0.214	0.250	0.321	0.286	0.214	0.179	0.179	0.250
GP2		0.107	0.179	0.179	0.286	0.357	0.321	0.321	0.214	0.143	0.214
GP3			0.214	0.214	0.179	0.393	0.286	0.357	0.250	0.250	0.250
GP4				0.214	0.393	0.464	0.500	0.286	0.321	0.107	0.393
GP5					0.250	0.393	0.357	0.286	0.250	0.179	0.321
GP6						0.286	0.321	0.393	0.286	0.357	0.214
GP7							0.250	0.321	0.286	0.429	0.357
GP8								0.357	0.250	0.393	0.250
GP9									0.179	0.250	0.393
GP10										0.214	0.214
GP11											0.357

Moumouni et al. (2022) found substantial genetic variability among the selected 18 elite cashew genotypes in Western Burkina Faso independently of geographic origins. The detected diversity might also arise from the predominance of entomophilous cross-pollination (Vanitha and Raviprasad 2019) and self-incompatibility (Eradasappa and Mohana 2019) in cashew.

The evaluated germplasm of cashew nut exhibited significant morphological and genetic variation in both qualitative and quantitative traits. The wide range of genetic

distance indicated the presence of genetic variability and the potentiality of RAPD markers for the identification of cashew germplasm for breeding. There had no direct relation between genetic diversity and the geographical location of cashew. So, the nut morphology and genetic variability could be used to select suitable parents for cashew improvement breeding programs.

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