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Genetic diversity analysis in cashew (*Anacardium occidentale* L.) germplasm using RAPD marker

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ABSTRACT

Anacardium occidentale L., commonly known as cashew nut, belongs to the family Anacardiaceae. It is regarded as a high valued fruit nut crop world-wide. Potential of this economically important nut is under-utilized in Bangladesh in spite of having all favorable agro-climatic conditions. The objective of the present investigation was to characterize six cashew accessions using Random Amplified Polymorphic DNA (RAPD) markers. Four random primers viz. OPE-02, OPE-18, OPK-03 and OPB-15 were used to amplify DNA segments. A total of 33 reproducible bands were obtained, out of which 11 were monomorphic and 22 were polymorphic. On average 74.12% polymorphism was observed. . Primers OPB-15 and OPK-03 yielded 100% polymorphism and OPE 02 and OPE 18 produced 33.33% and 63.16 % polymorphism, respectively. Cluster analysis revealed two main distinct groups, first group included GP-1 and the second consisted of five genotypes viz. GP-2, GP-3, GP-4, GP-5and GP-6 The major cluster- II was further subdivided into two minor clusters i.e. minor cluster- III and IV. Minor cluster- III contained only one genotype GP-4. Minor cluster- IV consists of four rest genotypes. The genetic distance between the groups was found low and varied from 0.002 to 0.0308. Maximum genetic distance was observed between GP-1 and GP-2cashew germplasm and minimum between GP-5 and GP-6. The low genetic distance which is unusual for this out crossing long-lived tree species, indicates the probability of having common ancestry among the germplasm or may be due to the use of a narrow range of populations for the investigation.

Introduction

Cashew (*Anacardium occidentale* L.) is an economically valuable tropical fruit tree crop (Rao and Swamy, 1994). The Anacardiaceae family includes about 75 genera and 700 species (Nakasone and Paull, 1998), of them cashew contains only 8 species (Azam-Ali and Judge, 2000). It is an evergreen perennial tree plant with dense foliage and can grow as high as 15 meters or more (Nakasone and Paull, 1998). The enlarged fleshy stalk of fruit is called cashew apple and the true fruit of the cashew tree is a kidney shaped drupe that grows at the end of the cashew apple. Cashew plant has been well known for its medicinal values. It has antifungal, antibacterial, antiseptic, and anti-inflammatory properties in its leaf and bark. Cashew is widely distributed throughout the tropics e.g. Brazil, Tanzania, Mozambique, Kenya, Sri Lanka, India and Vietnam. (Dasmohapatra *et al*., 2014). In India, cashew is grown in the Indian states of Kerala, Tamil Nadu, Karnataka, Andhra Pradesh, Odisha, Goa, Maharashtra, Madhya Pradesh, Manipur, Meghalaya, Tripura and West Bengal (Singh, 1996). Although having favorable agro-climatic situation in Bangladesh for cashew growing, potential of this economically important fruit nut has not been explored yet. Only a few cashew plantations are found in the hilly areas of greater Chittagong division. Cashew has been grown throughout the coastal region of India and Sri Lanka in Asia, except in Bangladesh where not a single cashew plantation in the coastal belt can be found. Overall fruit tree crop intensity is low in the coastal zones of Bangladesh; although there is an opportunity to introduce this economically valuable crop.

Each year Bangladesh imports a huge amount of processed cashew nut with high cost. Establishment of commercial cashew orchard in coastal areas is therefore a time demanding need of this country. Agrotechnology Discipline of Khulna University has started collecting the available germplasm of cashew and simultaneously has started characterization for their morphological and molecular traits.

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Molecular characterization and identification of genetic relationship is very important for maintaining landscaping and germplasm conservation of cross pollinated tree species. There are various types of molecular markers for genetic studies *viz.* Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Variable Number Tandem Repeat (VNTR), Simple Sequence Repeat (SSR), Single Feature Polymorphism (SFP), and Restriction-site associated DNA markers (RAD markers) etc. (Dhanaraj, 2002). Development of RAPD marker, generated by polymerase chain reaction (PCR) using arbitrary primers provided the cheapest and the easiest tool for detection of DNA polymorphism, leading to the determination of genetic relationship in plants (Nagori *et al*., 2018). Analysis of genetic relationship in cashew using RAPD banding data can be useful to plant improvement, description of varieties and also for the assessment of varietal purity in plant certification programs (Samal *et al*., 2003). In the present investigation, the diversity and genetic relationship of six cashew germplasm accessions were analyzed by using RAPD markers.

Materials and Methods

Plant materials

Six germplasm accessions of cashew plant were taken for the study. Those were GP-1, GP-2, GP-3, GP-4, GP-5and GP-6. The plant materials were collected from Germplasm Bank of Agrotechnology Discipline of Khulna University.

DNA extraction

The young leaves from the established plants were used for DNA extraction. The leaf samples were wrapped with aluminum foil paper and freeze-dried at -86 ºC in ultra low freezer for lyophilizing the sample. The leaves were finely ground in liquid nitrogen and approximately 200 mg of powdered leaf was taken in sterile 1.5 mL eppendorf tube. Norgenbiotek DNA isolation kit was used for DNA extraction. The purified DNA of leaves was quantified by using Spectrophotometer (Multiskan GO Microplate, Thermo Fisher Scientific, Germany) at 260 nm wave length. The final concentration of the template DNA was adjusted at 30 ng μL^{-1} for PCR and stored at -20 °C.

PCR reaction

Four 10-mer primers viz., OPE-02, OPE-18, OPK-03 and OPB-15 were used in this study (Table 1). Polymerase Chain Reactions were carried out in a 20 µL volume in 0.2 ml thin walled PCR tubes for each isolate. PCR reaction chemicals and their quantity are presented in Table 2. The amplification was achieved in a thermal cycler programmed for a 2 minutes of hot start at 94 °C, denaturation at 94 °C for 30 seconds, annealing at 37 °C for 1 minute and initial extension at 72 °C for 1 minute and repeat for 40 cycles, finally at 72 °C for 5 minutes and hold at 4 °C.

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

Sl no.	Primer Code	Sequence (5'–3')	GC Content (%)	Tm of Primer (°C)
1	OPE 02	GGT GCG GGA A	70.00%	30
2	OPE 18	TGC GGC TGA G	70.00%	30
3	OPB-15	GCA GGG TGT T	60.00%	30
4	OPK-03	CCA GCT TAG G	60.00%	30

Table 2. PCR reaction chemicals and their quantity

Sl no.	PCR reaction chemicals	Concentrations	Quantity
1	dNTPs	10 mM	0.2 µl
2	Taq DNA polymerase	5 U μl^{-1}	0.2 µl
3	Primer	100 pM μl^{-1}	1.0 µl
4	Reaction buffer without $MgCl_2$	10×	1.0 µl
5	$MgCl_2$	25 mM	1.2 µl
6	DNA template	30 ng μl^{-1}	1.0 µl
7	Molecular water (Sigma Aldrich, USA)		15.4 µl
Total			20.00 µl

Agarose Gel Electrophoresis for separation of PCR amplicons

The amplified products were resolved by electrophoresis on 1% agarose gels stained with ethidium bromide (0.5 µg mL^{-1}) run at 60 volts in Tris Acetate EDTA buffer (1.0 × TAE) for one hour. Resulted amplicon profiles of all the isolates across the primers were visualized under UV Tech Gel documentation System with computer program (Biometra gel documentation, A Biodoc Analyze 2.2 version, Germany). A molecular weight marker 1 kbp (Direct load, Sigma Aldrich, USA) was used.

Data analysis

The DNA profiles were scored visually from gel images. Clear and reproducible amplified bands were chosen in the analyses. The presence of a band was designated as (1) and absence as (0). The data obtained by scoring the RAPD profiles were subjected to the calculation of similarity matrix using Jaccard's coefficients (Zahra *et al*., 2012). The similarity values were used for cluster analyses. Sequential agglomerative hierarchical nonoverlapping (SAHN) clustering was applied using unweighted pair group method with arithmetic averages (UPGMA) method. Dendrograms were plotted using Restdist (version 3.69) computer programs by Joseph Felsenstein of the University of Washington, USA. Polymorphism percentage was calculated as the proportion of polymorphic bands over the total number of scored bands of respective primer.

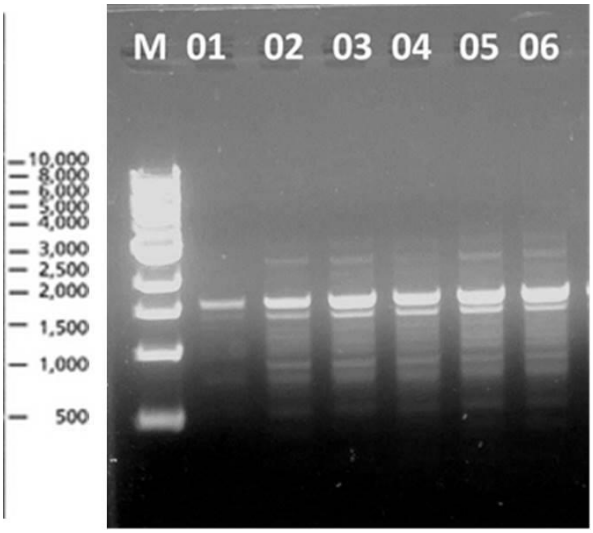
Results

Amplified DNA Banding Pattern

Among the amplified DNA products two types of bands were found *viz.* monomorphic and polymorphic bands. Those bands which present in all individuals are monomorphic; on the other hand, polymorphic bands are those which present in one or more but not in all individuals. The unique bands are only present in at least one individual (Mehetre *et al*., 2004). After RAPD analysis a total of 33 DNA bands were obtained from all the studied primers. Among them, 22 were polymorphic and 11 were the monomorphic. On an average, 74.12 % polymorphism was found. However, the average number of bands was 8.25 considering four primers. In case of primer OPE 02, the amplified DNA fragments ranged from 500 to 2500 bp. The amplified DNA fragments was found variable with the primer OPE 18 that ranged from 100 to 3500 bp. The primer OPB 15 produced bands of 250 to 750 bp size. In primer OPK 03, DNA fragments of 300 to 800 bp size were observed (Table 3, Figure 1 and Figure 2).

Table 3. Particulars of bands produced by four random primers in six cashew germplasm

Primer Code	Maximum Amplified Band	No. of Monomorphic Bands	No. of Polymorphic Bands	Polymorphism (%)	Range of DNA Fragments (bp)
OPE 02	06	04	02	33.33	500-2500
OPE 18	19	07	12	63.16	100-3500
OPB-15	04	00	04	100.00	250-750
OPK-03	04	00	04	100.00	350-850
Total	33	11	22	74.12 (Avg.)	

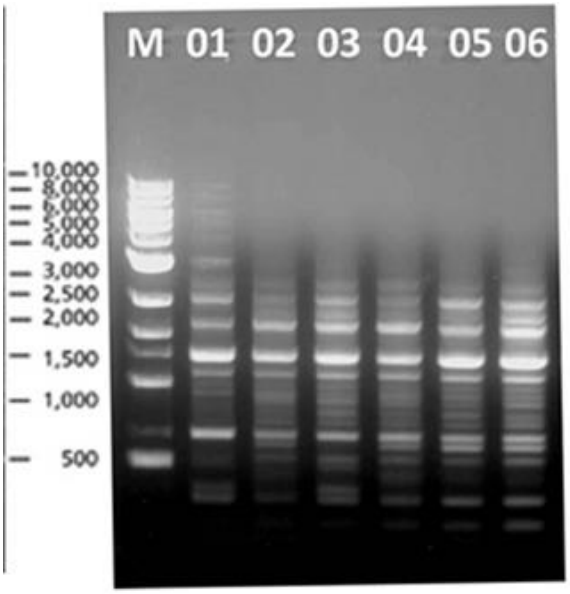


Number 01 to 06 indicates the germplasm number GP-1, GP-2, GP-3, GP-4, GP-5and GP-6 and "M" indicates the Ladder (1 kb)

Fig. 1 Gel image of amplified DNA fragments using primer OPE 02

In case of primer OPE 2, total 6 bands were amplified. Among them at 1600 bp one clear monomorphic band was observed (Figure 1). Another amplification was done by the using primer OPE 18. Here, a total of 19 DNA fragments were found.

Three clear monomorphic bands were observed. The clear monomorphic bands size were 700, 1600 and 2000 bp. In this case, two clear polymorphic bands were also found. The clear polymorphic bands sizes were 150 and 300 bp (Figure 2).



Number 01 to 06 indicates the germplasm number GP-1, GP-2, GP-3, GP-4, GP-5and GP-6 and "M" indicates the Ladder (1 kb)

Fig. 2. Amplified DNA fragments generated by using primer OPE 18

Cluster analysis

Two major clusters were found i.e. major cluster- I and major cluster- II from the cluster analysis of six selected cashew genotypes. Major cluster-I consists of only one genotype GP-1 and another 5 genotypes were belonged in major cluster- II. The major cluster- II was further subdivided into two minor clusters *i.e.* minor cluster- III and IV.

Whereas, the minor cluster- III contained only one genotype GP-4. Minor cluster- IV consists of four genotypes. Those four genotypes were further subdivided into two sub-minor clusters *i.e.* sub-minor cluster-V and VI. Only one genotype GP-2 was present in the sub-minor cluster-V. Sub-minor cluster-VI bearing three genotypes further divided into two groups *i.e.* group-VII and VIII. Group-VII has only one genotype GP-3 and group-VIII has two genotype GP-5 and GP-6 (Figure 3).

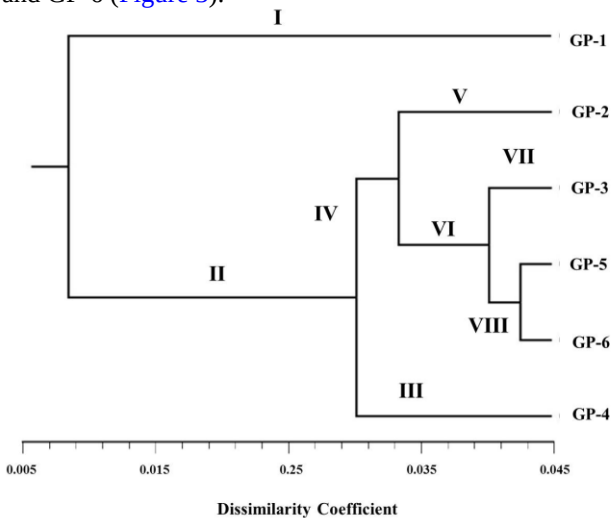


Fig. 3 Dendrogram generated from polymorphic data of four primers through RAPD analysis of six cashew genotypes

Distance Matrix

The correlation of genetic distance among the 6 genotypes is shown in the Table 4. Comparatively higher genetic distance was found between GP-1 and GP-2 which is 0.03. The lowest genetic distance was found between GP-5 and GP-6 which is 0.002. GP-5 and GP-6 genotype pair was very close to each other with the highest inter-varietal similarity index (99.8%) and the lowest genetic distance (0.002).

On the other hand, GP-1 to GP-2 pair was more distant to each other with the lowest inter-varietal similarity index (97%). The range of genetic distance of 6 genotypes was 0.002 to 0.03 and the highest and the lowest genetic distance indicated the presence of low variability among the genotypes of cashew.

Table 4. Genetic diversity index from RAPD data of different cashew germplasm

	GP-1	GP-2	GP-3	GP-4	GP-5	GP-6
GP-1	1.0000					
GP-2	0.0308	1.0000				
GP-3	0.0268	0.0105	1.0000			
GP-4	0.0288	0.0148	0.0133	1.0000		
GP-5	0.0299	0.0074	0.0028	0.0094	1.0000	
GP-6	0.0299	0.0097	0.0048	0.0096	0.0020	1.0000

Discussion

The study of genetic diversity of tropical tree species is useful to landscape management, plant genetic resource inventory and biological conservation. Conservation programs are already using data generated by molecular techniques to evaluate the genetic structure and diversity of natural populations. Genetic diversity changes due to particularly loss of diversity through loss of species, results in a loss of biological diversity. Changes in biodiversity result in changes in the environment, requiring subsequent adaptation of the remaining species.

In recent years, DNA profiling through RAPD technique has been used for the analysis of diversity and polymorphism within the large germplasm (Virk *et al*., 1995). In present study, the RAPD marker was applied to assess the genetic diversity among six cashew germplasm. Four random primers were used that produced 74.12% polymorphism on an average. Out of four primers used in this experiment, two produced 100% polymorphism and other two provided with 33.33% and 63.16 % respectively. Similar results with 74.86% polymorphism were reported by Samal *et al*. (2003) by analyzing 20 cashew genotypes using eleven RAPD primers. Dasmohapatra *et al*. (2014) reported 81.55 to 89.55% polymorphism by observing 25 cashew genotypes following RAPD technique. Thus, the per cent polymorphism of amplified DNA fragment may vary significantly in similar or different plant species. This is explicable as the product amplification depends upon the sequence of random primers and their compatibility with genomic DNA. The DNA bands resulted by each primer may be different in both size and amount of DNA bands. The present result is in agreement with Samal *et al*. (2003) findings.

In the present study, dendrogram was constructed based on UPGMA method which revealed two main distinct groups, among the six genotypes first group included GP-1 and the second group included the others. The genetic distance between the groups was only 3% that indicates probably they have some common ancestry. The existence of very low genetic diversity within the cluster I and cluster II of cashew germplasm may be attributed to self-pollination (Dhanaraj, 2002). Although cashew flowers are adapted to cross pollination, but sometimes in absence of pollen donor plants, insects may facilitate self-pollination. Among the cashew germplasm, the GP-4, GP-5 and GP-6 are the sibs of GP-2. And the GP-2 plant was grown as a single plant, any sorts of pollen donor plant was absent. Therefore low genetic diversity within these plants is expected (Kapinga *et al*., 2018).

Conclusion

A total of 33 reproducible bands and 74.12% polymorphism were observed. Primers OPB-15 and OPK-03 yielded 100% polymorphism respectively. Cluster analysis revealed two main distinct groups, first group included GP-1 and the second consisted of five genotypes *viz.* GP-2, GP-3, GP-4, GP-5and GP-6 The major cluster- II was further subdivided into two minor clusters i.e. minor cluster- III and IV. The genetic distance between the groups was found low and varied from 0.002 to 0.0308. Maximum genetic distance was observed between GP-1 and GP-2 cashew germplasm and minimum between GP-5 and GP-6. In spite of having some limitations such as use of limited number of germplasm, the result of this study could be used as a guideline for future diversity assessment and genetic analysis of cashew in Bangladesh. The analysis of genetic relationships in cashew using RAPD banding revealed that the method is appropriate enough to evaluate the genetic relationships in cashew nut tree and thus the RAPD markers can generate wide array of polymorphism for varietal identification, study of genetic diversity and genetic conservation, and could be successfully used in cashew breeding program.

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