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Molecular Marker (Rapd) Based Fingerprinting on Jackfruit to Estimate The Genetic Diversity

Gopalsamy J[1*], Anburaj J[1], Sundaravadivelan C[2], Kuberan T[3,] Kumar P[2], Starlin T[4] and Mariselvan M[5]

[1]Post – Graduate & Research Department of Biotechnology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, India
[2]Post – Graduate & Research Department of Zoology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, India
[3]Post – Graduate & Research Department of Microbiology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, India
[4]Post – Graduate & Research Department of Bioinformatics, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, India
[5]Post – Graduate & Research Department of Zoology, St. Xavier's College, Palayamkottai, India.

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Jackfruit (*Artocarpus heterophyllus* Lamk.) belongs to the family Moraceae. It grows abundantly in Bangladesh, India and in many parts of Southeast Asia. The fruit has been reported to contain high levels of protein, starch, calcium and thiamine. In Bangladesh, it is commonly referred to as "poor man's food". Many parts of the plant including the bark, roots, leaves and fruit, have medicinal properties. The leaf samples of the selected jackfruit varieties were collected, air dried and powdered using liquid nitrogen and was found suitable for further isolation. High quality of DNA getting in five varieties of jackfruit viz.TCJ4, TCJ2, FH10, FH4, and NC2. Amplification of jack fruit DNA for RAPD analysis using OPC7-GTCCGACGACGA was analyzed. The PCR reaction mixture was found optimum to produce intense and reproducible banding patterns in jack fruit. The dendrogram based on Euclidean distance was constructed using the computer package 'STATISTICA'. This dendrogram indicated a moderate diversity among the five jack fruit varieties. Three major clusters were identified, one cluster with 2 varieties. The individuals in a cluster share common phylogenetic characteristics. This investigation to enables the identification and selection of jack fruit varieties for breeding programs and effective germplasm management.

Artocarpus heterophyllus / Genetic diversity

Jackfruit (*Artocarpus heterophyllus* Lamk.) belongs to the family Moraceae. The tree is cultivated widely at low elevations through out India, Burma, Sri Lanka, Southern China, Malasiya, the East Indies, Bangaladesh, and in many parts of Southeast Asia (Rahman et al. 1999). Jackfruit is also present in several other countries, including evergreen forest zone of West Africa (Burkill, 1997).

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**To whom correspondence may be addressed. Email: geneanbu@yahoo.co.in

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Jackfruits a medium sized ever green tree, typically reaching 8-25 m in height that is easily recognized by its fruit, in the largest amount of all cultivated plants. Jackfruit are generally classified into main types, those having fruits with small, fibrous, soft and spongy flakes with very sweet carpals and other type being crunchy though not as sweet with crisp carpals and of high quality. Cultivar identification and estimates of genetic diversity using phenotyphic markers have several limitations, especially in perennial crops (Odoemelam, 2005). The succulent, aromatic and flavorful fruits are eaten as fresh or preserved in many forms. The fruit has been reported to contain high levels of protein, starch, calcium and thiamine. In Bangladesh, it is commonly referred to as "poor man's food" (Rahman et al. 1999). The nutritious seeds are boiled or roasted and eaten like chestnuts, added to flour for baking and cooked in dishes. The tree is also known for its durable timber. This ages to on orange or reddish brown color with antitermite properties. The leaves and fruit wastes provide valuable fodder for cattle, pigs and goats. Many parts of the plant including the bark, roots, leaves and fruits, have medicinal properties. Jackfruit wood chips yield a dye, which is used to give the famous orange red color to the robes of buddies priests (Criag and Harley, 2006). The Chinese consider jack fruit pulp and seeds to be a tonic, cooling , nutritious and useful in over coming the influence of alcohol.

Jack is a minor fruit crop having multifarious user products like pickles, de hydrate, leather, canned bulbs and jelly, pap ads can be prepared. The seed is rich in nutritive values and used in many Colin any preparation the rind or skin of the fruit and leaves of the plant can serve as excellent cattle feed in India though this plant is grown as a back yard crop; it is not commercially much exploited. The seed starch is given to relieve from biliousness and the roasted seed are regarded as on aphrodisiac. The ash of jack fruit burnt with corn and coconut shells is used either alone or mixed with coconut oil to heal ulcer. The dried latex yields artostenone, convertible to artostenone, a compound with marked androgen action while the latex mixed with vinegar promotes the healing of abscesses, snakebites and glandular swellings. The root is used as a remedy for skin disease and asthma. An extract of the root is taken in cases of fever and diarrhea, and the bark is made into poultices. The wood has a sedative property, while its pith is said to cause abortion.

A molecular marker is any measure character and molecular characteristic that is inherited in a simple mendalian fashion. The discovery of molecular markers in recent years has greatly enhanced the scope for detailed genetic analysis and approaches to improve crop plants. The molecular technology has indirectly improved the efficiency of plant breeding programs. Molecular markers play two main roles in plant breeding programs, firstly as a source of genetic finger prints and as a selected marker linked to phenotyphic traits of interest to breeder. Markers are broadly classified into morphological markers, protein based markers and DNA based markers. The recently developed DNA based marker like, Restriction fragment length polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) and Amplified fragment length polymorphism (AFLP) can act as excellent tools to study the genetic diversity eliminate duplicate in germplasm to study the genetic relationships, gene tagging, genome mapping and to use in PVR (plant variety rights) purpose these markers measure, diversity at DNA level in all tissue at all of plant and are seldom influenced by environmental condition

Molecular differences using DNA and protein based markers are more authentic and unaffected by environmental factors (Dhanraj et al. 2002). Hence, characterization of genotypes at the genetic level supplemented by phenotypic characters and it provides the first step towards more efficient conservation maintenance and utilization of existing genetic diversity (Prakash et al. 2002). Among DNA based molecular markers Random Amplified Polymorphic DNA's (RAPD) provide an excellent tool for studying genetic diversity and genetic relationships (Williams et al. 1990).They are simple versatile relatively inexpensive to detect slight genetic differences and help to identify duplicates in the population. RAPD markers have been used successfully to study genetic diversity and relatedness among perennial fruit crops such as mango (Ravishankar et al. 2000). In this present study OPC7(GTCCGACGACGA) RAPD marker were used to estimate genetic diversity and assess the relationship among 5

jackfruit accessions from different parts of southern India maintained at University of Agricultural Sciences, Bangalore. This investigation enables the identification and selection of jack varieties for breeding programs for crop improvement and effective germplasm management.

Materials and Methods

The five different varieties of (TCJ4, TCJ2, FH10, FH4 and NC2) *Artocarpus heterophyllus* were collected from Horticulture Farm, Division of Horticulture Science, GKVK, Bangalore. Five different varieties of leaf sample *Artocarpus heterophyllus* were collected in brown paper covers and brought to the laboratory. Leaves were washed with tap water and air-dried. The leaves were then sealed in airtight plastic bags, labeled and stored at 4˚C for further studies.

Isolation of DNA

The total genomic DNA was isolated from leaves of five different varieties of Jack fruit genotypes (TCJ4, TCJ2, FH10, FH4, NC2) using CTAB method (Porebski et al. 1997). One gram of leaf tissue was ground to fine powder using mortar and pestle in liquid nitrogen. Then the sample is transferred in to extraction tube with 20ml of CTAB buffer (pre-heated for about 5-10 min in hot water bath at 65°C) each tubes mixed thoroughly by inverting the tubes. The tubes were incubated at 65° C for 1 hour in hot water bath with intermittent shaking and brought to temperature. Then 6ml of chloroform : isoamyl alcohol (24:1)was added to the each tube and gently mixed for 25-30 times by inverting the tube gently and spin at 7000rpm for 10-15min at4°C. The supernatant was transferred to a fresh centrifuge tube and the procedure was repeated to get a clear form of supernatant. To the supernatant half the volume of 5M NaCl and one volume of ice cold propanol was added and incubated overnight at 4°C. The content was centrifuged at 10000rpm for 15 minutes at 4°C.The resulting supernatant was discarded and the pellet was washed with 70% ethanol and air dried. The pellet was finally dissolved in 300µl of TE Buffer .The quality of DNA was checked by 0.8% agarose gel electrophoresis. The electrophoresis was carried out at 80V for 1 hour 30minutes.

Purification of isolated DNA: The isolated DNA sample was purified by treating with 5µl of RNase and 5µlof proteinase k and incubated at 37° C for one hour and 300µl of phenol was added. The mixture was centrifuged at 7000 rpm for 10 minutes and the supernatant was transferred in to fresh tube. To the supernatant 300µl of phenol: chloroform (1: 1) volume was added and centrifuged at 7000 rpm for 10 minutes at 4°C.The resulting supernatant was again transferred to fresh tubes and 300 µl of chloroform was added and centrifuged at 7000 rpm for 10 minutes at 4° C. Half the volume of 5 M NaCl and one volume of ice cold propanol were added to the supernatant and incubated overnight at 4° C. The tubes were centrifuged at 10000rpm for 20 minutes and the supernatant was discarded. The pellet was washed with 70% ethanol and dissolved in 300µl of TE Buffer. The quality of DNA was checked in 0.8% agarose gel electrophoresis. The electrophoresis was carried out at 80V for 1 hour 30minutes.

The isolated and purified DNA was checked for quality and quantity prior to PCR amplification. The quality of the DNA was checked using 0.8% agarose gel electrophoresis. The isolated and purified DNA was analysed quantitatively by using UV spectrophotometer. The OD value was measured at 260nm and 280nm.

$$\text{Quantification} = \frac{\text{O.D. at 260nm} \times 50\ (\text{Dilution factor}) \times 50\mu\text{g/ml}}{1000}$$

PCR amplification

PCR amplification was carried out by following the modified method of Williams et al. (1990). The primer (OPC7-GTCCCGACGA) was screened to using pooled jack fruit DNA and distinguishable bands and were selected for further PCR analysis. The reproducibility of the primer was tested by repeating the PCR amplification three times under similar conditions. Each PCR reaction was carried out 25µl containing template DNA, dNTPs, $MgCl_2$, Taq DNA polymerase (Sigma Aldrich chemicals, Bangalore, India), primer and PCR buffer. Amplification was performed in Thermocycler. Programmed for an initial denaturation at 94˚C

for 4min. followed by 45 cycles of denaturation at 94˚C for 1min. Annealing at 35˚c for 1min, primer extension at 72˚c for 2min and final extension at 72˚c for 10min.PCR products were resolved in 0.8% agarose gel, visualized and documented using an Alpha digide system. The PCR reaction was carried at in a final volume of 25µl reaction mixture contained. The mixture was over layered with a drop of mineral oil. The PCR reaction was repeated twice using the same conditions to check the repeatability of amplified products.

Diversity analysis: The diversity analysis was carried out using the cluster analysis computer package 'STATISTICA'. Amplified fragments from each RAPD primer (OPC7-GTCCCGACGA) were scored manually for their presence or absence and a RAPD matrix of the 5 accessions of jack fruit using one primer (OPC7-GTCCCGACGA) was assembled for statistical analysis. The size of the fragments was estimated using 1kp standard DNA ladder electrophoresis with the products. A genetic dissimilarity matrix was developed using Squared Euclidean distances. This estimates all pair-wise differences in the amplification products (Sokal and Sneath, 1973).A cluster analysis was based on ward's method using a minimum distance algorithum (Ward, 1963).

Results and Discussion

Jackfruit is one of the most important plantation crops in India. It is cultivated in many states of India. Jack fruit has many industrial and medicinal properties. Considering the importance of this crop, there is a prime need for its improvement through breeding. Information on nature and genetic variability of the germplasm collection is very essential for crop improvement. The RAPD characterization of five different varieties of Jack fruit *Artocarpus heterophyllus* Lamk. were studied. The standardized protocol involved the DNA carried out with 2 percent PVP and CTAB resulted high yield of DNA. The yielded DNA was adequate for RAPD analysis. Jackfruit leaves were moderately rich in phenols and carbohydrates especially in the recently matured leaves which were rich in carbohydrates and other secondary metabolites. The use of higher amount of PVP and CTAB were used which would remove phenol, polysaccharides and other secondary metabolites (Schnell et al. 2001). Using recently matured leaves resulted in dull, white translucent DNA pellets, which were easily dissolved in TE buffer. The DNA purified by using this protocol was homogenous and not degraded.

Extraction treatment was followed during purification to obtain quality of DNA, which should be free from RNA and protein. The presence of contaminants like proteins, phenols, polysaccharides and other secondary metabolites inhibit the enzyme action in PCR purification has to be done to get clear DNA which consistently amplifiable in polymerase chain reaction. The most commonly used methods for deproteinizing solution of DNA was extraction with phenol, which sufficiently denatured protein. To remove polyphenols three per cent of PVP was used while extracting DNA of *Artocarpus heterophyllus* which formed a complex with polyphenols through hydrogen bonding allowing them to get separated from the DNA. Extraction of DNA with three per cent CTAB helped in reducing the polysaccharide level (Porebski et al. 1997). Extracted DNA is viewed along with the RNA and Protein contamination (Plate 1) and purified DNA is viewed without RNA and Protein contamination (Plate- 2).

The aim of any genomic DNA isolation was to isolate DNA of high molecular weight with sufficient purity. The presence of contaminants in the isolated DNA would inhibit the enzyme action in PCR. Hence the purity of DNA was checked by calculating the A_{260}/A_{280} ratio which lied between 1.4 and 1.6. This proved to be highly purified DNA (Table- 1). The PCR amplification was performed by optimizing the concentrations of template DNA, primer and Taq polymerase. Amplification was carried out by subjecting the samples at three temperatures corresponding to denaturation, annealing and extension. The cyclic regime of 45 cycles with a hot start of 95°C for 2 min and each cycle consisting of denaturation at 94°C for 1 min. Denaturation step that was too long leads to the unnecessary of enzyme activity. Annealing at 45°C for 1 min and annealing for a period of one minute was found effective of good banding pattern. The temperature and length of time required for primer annealing depend upon the base composition of the primer. Extension at 72 °C for 3 min with a final

extension of 72°C for 5 min, were found optimal for the amplification of jackfruit DNA (Plate 3).

Table -1: Quantification of DNA

S. No	Samples	OD Value at 260nm	OD Value at 280nm	Average OD(260/280nm)
1	TCJ4	0.137	0.297	1.43
2	TCJ2	0.138	0.087	1.58
3	FH10	0.118	0.077	1.53
4	FH4	0.127	0.089	1.42
5	NC2	0.140	0.097	1.44

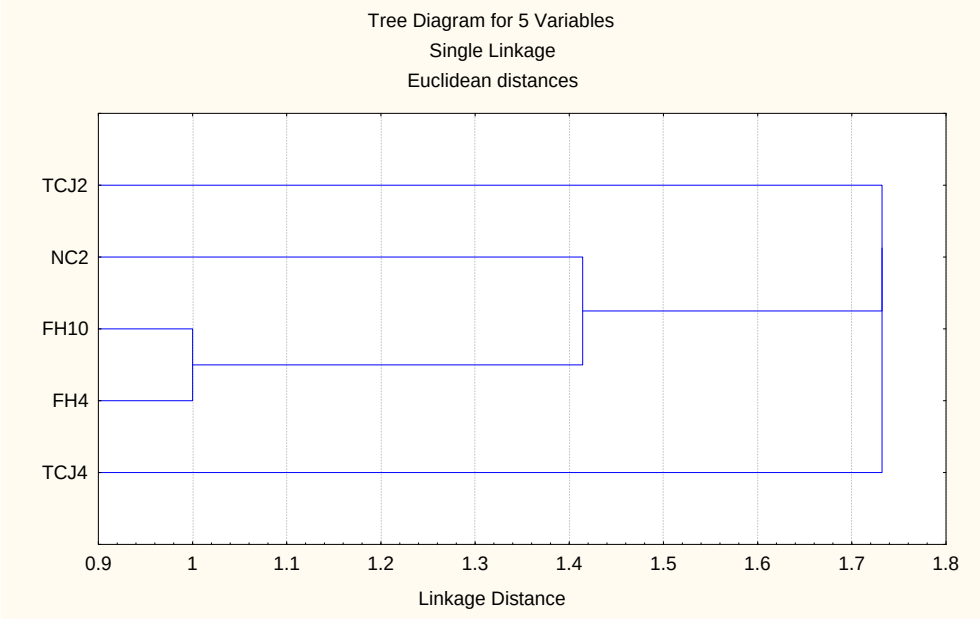
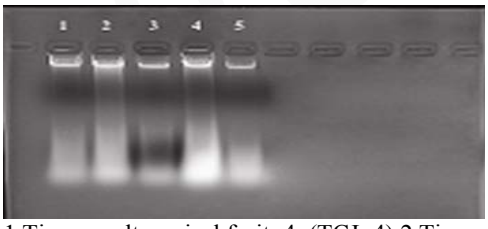
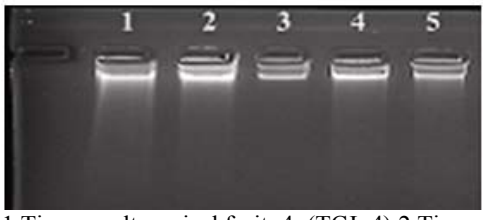


Fig 1. Dendrogram analysis selected five jackfruit varieties using STATISTICA



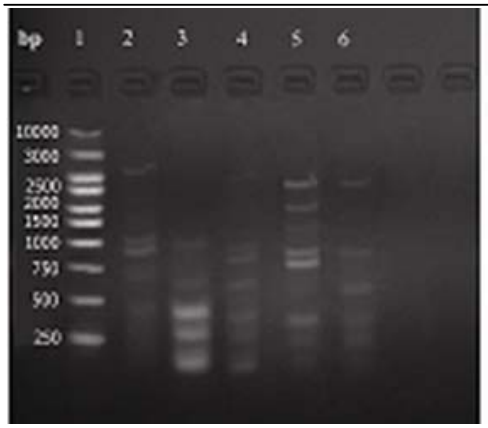
1.Tissue culture jackfruit 4 (TCJ 4);2.Tissue culture jackfruit 2 (TCJ 2); 3. Farm house 10 (FH 10); 4.Farm house 4 (FH 4); 5.Non cracking 2 (NC 2)

Plate- 1: Identification of isolated DNA from five different Jackfruit (*Artocarpus heterophyllus*)



1.Tissue culture jackfruit 4 (TCJ 4);2.Tissue culture jackfruit 2 (TCJ 2); 3. Farm house 10 (FH 10); 4.Farm house 4 (FH 4); 5.Non cracking 2 (NC 2)

Plate -2: Isolated and purified DNA from five different Jackfruit (*Artocarpus heterophyllus*)



1.1kb ladder; 2. Tissue culture jackfruit 4 (TCJ 4);3.Tissue culture jackfruit 2 (TCJ 2);4.Farm house 10 (FH 10);5.Farm house 4 (FH 4);6.Non cracking 2 (NC 2)

Plate-3: RAPD PCR profiles of jackfruit (*Artocarpus heterophyllus*)

The reaction mixture of 5 µl of template DNA, 2 µl of dNTPs and 1 µl of Taq polymerase were found to be optimum to produce intense and reproducible banding patterns in jack fruit. As the DNA concentration was increased, the number of fragments obtained also increased while too much DNA produced either a smear effect or did not amplify any fragments. The optimum concentration of dNTPs gave increased specificity and adequate amplification of DNA. This preliminary screening facilitated the selection of primers producing a higher level of polymorphism and more reproducible DNA fragments (Williams et al. 1990). The reproducible banding profile in *Artocarpus heterophyllus* DNA concentration in templates, concentration of different in reaction mixture as well as cycling conditions was varied. The result suggested a template DNA concentration of 5ng/µl reaction gave a consistent banding pattern. The two factors that mainly affected the strength of PCR amplification were the magnesium concentration and the annealing temperature. It was found that magnesium concentration of 15Mm was optimum. Since lower concentration resulted in less intense bands and higher concentration resulted in either a smear or no amplification. An annealing temperature of 35ºC was selected, since at this temperature reliable amplification of DNA was obtained utilizing Operon random primers of 10 base pairs length. A concentration of 100µm dNTPs was optimum. The higher concentration of dNTPs resulted in smears and low concentration gave faint bands or on fragments. A pair-wise genetic similarity and dissimilarity matrix was calculated using squared Euclidian distances method. The present analysis on genetic diversity among the jack fruit varieties are based on the amplified fragments by the primer OPC7. In the dendrogram (Figure 1), all the five varieties were grouped into three clusters 'A', 'B'and.'C'. Cluster A has varieties TCJ2 and TCJ4 which are closely related. The highest genetic similarity between clones of TCJ2 and TCJ4 is 1.75%. Cluster B has varieties FH10 and FH4 which are closely related. The lowest genetic similarity between clones of FH10 and FH4 is 1%. Cluster C has varieties NC2, is distanously related from the remain four varieties. The lowest genetic dissimilarity was NC2 is 1.42%.

Amplified fragments were scored by visual observation for their presence or absence of bands on the gel. The sizes of the fragments (molecular weight in base pairs) were estimated by using 1kb ladder, which was run along with the amplified products. The linkage distance was calculated according to Euclidean distance and cluster analysis. This cluster analysis grouped the varieties into 2 clusters which are closely related. Thus the cluster analysis helped to analyze the genetic diversity among the selected jackfruit varieties. Lukesimen et al. 2007 suggested that genetic dissimilarity matrix was calculated based on squared Euclidian distance which revealed maximum genetic distance of 7.9% between a clone of mottovarica (Mo), Chandrahalasu from distant location, while the minimum genetic distance (5%) was between the genotypes (Mo) and Kerala indicating the similar geographical origin. Wards method of cluster analysis group all genotypes on the dendrogram in to major clusters according to their geographical location and above study shows that, low to moderate genetic diversity among the 12 jackfruit accessions, which will assist in the identification and management of jackfruit germplasm for breeding purposes. Although the jackfruit accessions in the present study were collected based on their variable fruit morphology, they showed relatively low polymorphism, regardless of the location from which they are obtained from the western hats. Although this study has shown close

relationship among the genotypes, there were no duplicates. Cluster analysis helped to group genotypes with similar morphological features according to their original location, as well as depicting their pulp character. Amplification produced by primer OPC7 could segregate accessions according to cluster. This study confirmed the value of RAPD markers in estimating genetic diversity in jackfruit. This would be a first step towards more efficient germplasm storage and management in perennial crops, where land, time, effort and money could be saved (Lukesimen et al. 2007).

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