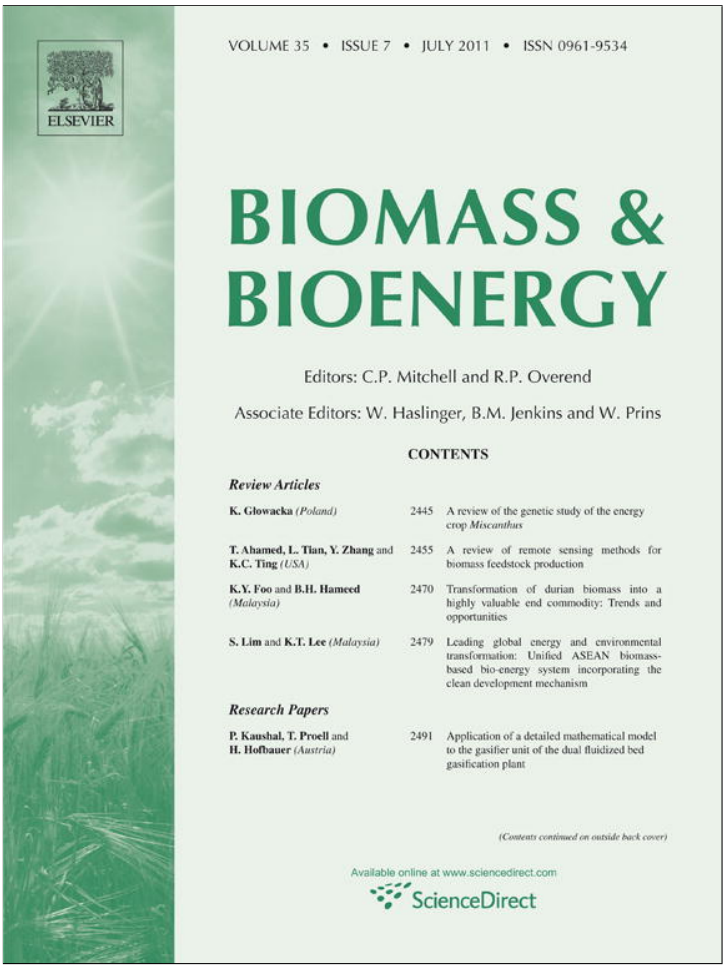

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# Genetic diversity assessment of *Jatropha curcas* L. germplasm from Northeast India

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ABSTRACT

Genetic variability in the wild genotypes of *Jatropha curcas* L., collected from different parts of Northeast India, was analyzed using two different single primer amplification reactions (SPAR) methods, *viz.*, inter-simple sequence repeats (ISSR) and directed amplification of minisatellite DNA (DAMD). A total of 36 genotypes were used to investigate the existing natural genetic variation at intra-specific level. One hundred forty nine (149) amplification products were scored by ISSR and DAMD, both of which collectively showed 75.83% polymorphism with a mean intra-population genetic diversity ($H_S$) of 0.1309. However, their level of diversity at inter- and intra-population levels was significant, with the percentage of polymorphic loci (P) ranging from 22.82% to 44.30%, Shannon's information index ($H_{pop}$) from 0.1302 to 0.2541 and Nei's gene diversity ($H_E$) from 0.0831 to 0.1723 with mean Nei's gene diversity ($H_T$) 0.2202 and the overall estimate of gene flow being (N*m*) 0.8085. Analysis of molecular variance (AMOVA) showed that 68.88% of variation at intra-population level, whereas 31.12% variation was recorded at inter-population level. Cluster analysis also supported the existence of genetic diversity in the genotypes of *J. curcas* collected from Assam and Meghalaya provinces of Northeast India. Present investigation suggests the efficiency of SPAR methods to estimate the genetic diversity precisely which can define genetic relationship and population genetics of *J. curcas*.



## 1. Introduction

*Jatropha curcas* L. (Euphorbiaceae) commonly known as '*physic nut*' is a multipurpose tree of Latin American origin, distributed throughout the tropical and subtropical regions of the World including India [1]. *J. curcas* has been found to be highly promising species in terms of oil yield, which can be used as a source of energy in the form of biodiesel [2]. It is a highly medicinal, economically valuable and well-known as energy crop throughout the World [1–5]. It is used for preparation of the ayurvedic drug ''*dravanti*'' [3] and is a potential source of lubricants, soaps, candles and coloring dye besides being useful as purgative, emetic, abortifacients and astringents [4,5]. The economic importance of *J. curcas* generates a wide interest among researchers to evaluate the existing natural genetic diversity, for selection and breeding of superior genotypes both at inter- and intra-specific levels. Analysis of genetic diversity using molecular markers is a trusted and reliable approach which could provide useful baseline information for breeding programs of various wild as well as cultivated crops [6]. Among the various molecular markers employed to assess diversity studies, PCR-based markers such

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as RAPD (Random Amplified Polymorphic DNA) [7,8], ISSR (Inter-simple Sequence Repeats) [9], DAMD (Directed Amplification of Minisatellite DNA) [10] and AFLPs (Amplified Fragment Length Polymorphism) [11] are popular, as their application does not need any prior sequence information. On the other hand, microsatellite or simple sequence repeats (SSR), the markers for breeding applications, are quite expensive and time consuming [12]. Although various approaches have been carried out by researchers to assess genetic variation in populations of *J. curcas* [13–18], but not much work has been carried out from the populations of Northeast India. Ranade et al. [15] in their study have indicated that two accessions of Northeast India are genomically different as compared to those occurring in other parts of India. Besides this, the seed oil content of various *J. curcas* genotype was reported to exhibit variation in the range of 25.63–42.46% which is quite significant and reflect the underlying genetic variability (unpublished data). Therefore, the present study was taken up to assess and analyze the natural genetic diversity existing among *J. curcas* genotypes at intra-specific levels representing Assam and Meghalaya.

## 2. Materials and methods

### 2.1. Study sites and sample collection

Six populations inhabiting different regions *viz.*, Ri-Bhoi (R), West Garo Hills (WG), and South Garo Hills (SG) districts from Meghalaya, and Kamrup-Metro (KM), Nagaon (NG), and Sonitpur (SP) districts from Assam (Table 1; Fig. 1; Table S1) provinces of Northeast India were selected. Six individuals per population (36 individuals) were randomly selected for present study. Leaf samples from these plants were collected and immediately frozen in liquid nitrogen until DNA extraction was performed.

### 2.2. DNA isolation and PCR amplification

Total genomic DNA was extracted from young leaves following the standard CTAB method with few minor modifications [19]. Genomic DNA was quantified against a known quantity of unrestricted Lambda (λ) DNA by electrophoresis using 0.8% agarose gel.

### 2.3. PCR optimization and primer survey

Amplifications with ISSR and DAMD primers containing varying concentrations of (i) template DNA (20–60 ng), (ii) Taq DNA polymerase (0.5–2 U), and (iii) $Mg^{++}$ salt (0–5 mM) were used to optimize reaction conditions of the Polymerase Chain Reaction (PCR). Of the five different concentrations of template DNA, 50 ng was found to be the most ideal as it yielded maximum number of reproducible bands. In addition, 1.5 mM of $MgCl_2$, and 0.6 U Taq DNA polymerase have given ideal results among 90 various concentrations tested. Thirty ISSR (Operon Technologies, USA) and 8 DAMD (Bangalore Genei, India) primers were assayed to identify primers which yielded reproducible and polymorphic patterns.

### 2.4. ISSR and DAMD amplification reactions

All the PCR reactions were carried out in 25 μl volumes containing 50 ng of template DNA, 200 μM of each of the four dNTPs, 1X PCR buffer (10 mM Tris pH 9.0, 50 mM KCl), 1.5 mM $MgCl_2$, 0.6 U Taq DNA polymerase (Bangalore Genei, India) and 10 pmol of primer (in case of ISSR) and 20 pmol (in case of DAMD). The reaction programmes were set at 94 °C for 4 min followed by 40 cycles of 92 °C for 30 s, 1 min at annealing temperature (42–58 °C depending on the primer's Tm and/or according to GC contents), 2 min elongation at 72 °C and a final extension at 72 °C for 7 min in a thermal cycler 2720 (Applied Biosystems, USA). For DAMD amplification, reactions were carried out at 94 °C for 2 min followed by 40 cycles of 92 °C for 1 min, 2 min at 55 °C, 2 min elongation at 72 °C and a final extension at 72 °C for 5 min [20]. After completion of the amplification, 2.5 μl 10X blue dye was added to the samples, and the amplified DNA was analyzed on 2% agarose gel in 1X TAE buffer at 65–70 V for 4–5 h.

### 2.5. Scoring and data analysis

Only clear and non-ambiguous amplicons were scored across all samples. These fragments were scored independently as either present (1) or absent (0) in each population and a binary data matrix was constructed. Molecular weights of the bands were estimated by using 0.5 kb and 0.1 kb DNA ladders (Bangalore Genei, India) as standards for DAMD and ISSR respectively. The ISSR and DAMD-PCR fragments were analyzed as alleles, under the following assumptions. Firstly,

**Table 1 – Details of populations of *J. curcas* sampled.**

| Assigned name of Population | Area of plant collection | No. of plants collected per population | Collection name | Latitude (N) | Longitude (E) | Altitude (M) |
|---|---|---|---|---|---|---|
| Meghalaya | | | | | | |
| Pop1 | Ri-Bhoi | 6 | SK-RB1 to SK-RB6 | 25°51′41.82″ | 91°52′49.74″ | 537.0 |
| Pop2 | South Garo Hills | 6 | SK-SG1 to SK-SG6 | 25°12′20.22″' | 90°18′44.88″ | 82.5 |
| Pop3 | West Garo Hills | 6 | SK-WG1 to SK-G6 | 25°26′37.62″ | 90°12′37.56″ | 277.9 |
| Assam | | | | | | |
| Pop4 | Kamrup-Metro | 6 | SK-KM1 to SK-KM6 | 26°6′16.56″ | 92°0′12.48″ | 60.1 |
| Pop5 | Nagaon | 6 | SK-NG1 to SK-NG6 | 26°25′31.80″ | 92°50′57.42″ | 62.3 |
| Pop6 | Sonitpur | 6 | SK-ST1 to SK-ST6 | 26°39′37.86″ | 92°49′47.94″ | 59.3 |

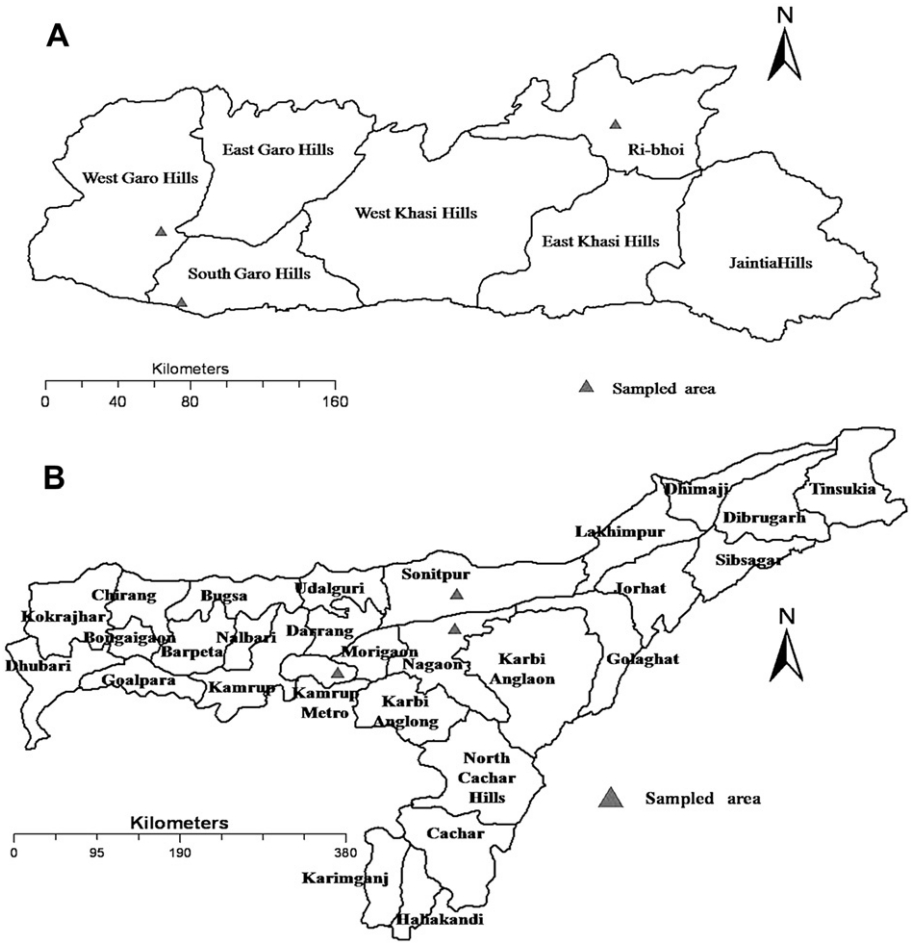


**Fig. 1 – Details of populations of *J. curcas* sampled (A) Meghalaya, and (B) Assam.**

ISSR and DAMD products segregate as dominant alleles in a Mendelian fashion. Secondly, the groves in this study were in Hardy–Weinberg equilibrium. Finally, the ISSR and DAMD fragments represented the nuclear genome and fragments of the same apparent size in different trees were homologous. These allele frequencies were calculated from ISSR and DAMD band frequencies following the methods and corrections employed by Lynch and Milligan [21]. We used POPGENE 1.31 [22] to calculate a set of intra- and inter-population genetic parameters, including genetic diversity at intra-populations level ($H_S$), genetic diversity at inter-populations level ($D_{ST}$) and the relative magnitude of genetic differentiation at inter-population levels ($G_{ST} = H_T - H_S/H_T$). Based on the island model, gene flow was inferred indirectly using Wright's [23] formula: $Nm = 0.5(1 - G_{ST})/G_{ST}$. Shannon's indices [24] were also calculated and used to characterize the gene diversity and distribution of the variation based on the formula $H_0 = -\sum$ pi $\log_2$ pi, in which pi is the frequency of a given ISSR and DAMD fragment. $H_0$ was calculated at two levels: the average diversity at inter-populations level (*Hpop*) and the total diversity (*Hsp*). The proportion of diversity at inter-populations level was estimated as (*Hsp* – *Hpop*/*Hsp*). Following Nei using corrected allele frequencies [25], Nei's gene diversity ($H_E$) was calculated. An analysis of molecular variance (AMOVA) [26] was performed to examine variability inter and intra-population level. Input data files for the AMOVA v. 1.55 program [26] were generated using AMOVA-PREP [27]. The variance components were tested statistically by nonparametric randomization tests using 1000 permutations. Data were analyzed using simqual route to generate Jaccard's similarity coefficient with NTSYS pc Versions 2.02k programme [28]. Dendogram was prepared for all the individuals using UPGMA and SAHN clustering for analysis of genetic relationships for all the collections.

## 3. Results

### 3.1. *SPAR analysis and profile polymorphism*

#### 3.1.1. *ISSR–PCR*

Fourteen ISSR primers produced 102 fragments, of which 70 were polymorphic (68.62%). The amplified fragments ranged from 0.2 to 3 kb with an average of 7.28 fragments per primer. The primers A1, A2, A5, and A8 exhibited the highest level of polymorphism and the percentage of polymorphic bands was calculated as 85.71, 90.90, 88.88 and 100% respectively (Table 2; Fig. 2A). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.73 to 0.95 (Table 3).

**Table 2 – Data of ISSR and DAMD primers used in the present study and the extent of polymorphism.**

| Sl. No | Primer's name | Primer's sequence (5′-3′) | Total No. of bands amplified | No. of polymorphic bands | No. of mono-morphic bands | % of Polymorphism |
|---|---|---|---|---|---|---|
| ISSR | | | | | | |
| 1 | A1 | AGAGAGAGAGAGAGAGYT | 7 | 6 | 1 | 85.71 |
| 2 | A2 | ACACACACACACACACG | 11 | 10 | 1 | 90.90 |
| 3 | A3 | GAGAGAGAGAGAGAGAYC | 8 | 4 | 4 | 50 |
| 4 | A4 | ACACACACACACACACYA | 8 | 5 | 3 | 62.5 |
| 5 | A5 | GAAGTGGGGAAGTGGG | 9 | 8 | 1 | 88.88 |
| 6 | A6 | HBHAGAGAGAGAGAGAG | 7 | 3 | 4 | 42.85 |
| 7 | A7 | BHBGAGAGAGAGAGAGA | 8 | 4 | 4 | 50 |
| 8 | A8 | ACCACCACCACCACCACC | 3 | 3 | 0 | 100 |
| 9 | A9 | AGAGAGAGAGAGAGAGCC | 8 | 5 | 3 | 62.5 |
| 10 | A10 | TGTGTGTGTGTGTGTGGG | 8 | 5 | 3 | 62.5 |
| 11 | A11 | CACACACACACAAC | 7 | 3 | 4 | 42.85 |
| 12 | A12 | CACACACACACAGG | 8 | 6 | 2 | 75 |
| 13 | A13 | GTGTGTGTGTGTGG | 5 | 5 | 0 | 100 |
| 14 | A14 | CACACACACACAGT | 5 | 3 | 2 | 60 |
| DAMD | | | | | | |
| 15 | HVR[a] | CCTCCTCCCTCCT | 10 | 10 | 0 | 100 |
| 16 | M13[b] | GAGGGTGGNGGNTCT | 8 | 8 | 0 | 100 |
| 17 | YNZ22[c] | CTCTGGGTGTCGTGC | 10 | 10 | 0 | 100 |
| 18 | INS[c] | ACAGGGGTGGGG | 8 | 4 | 4 | 50 |
| 19 | PER1[d] | GACNGGNACNGG | 11 | 11 | 0 | 100 |

A/C/G/T = N, A/C/T = H, C/G/T = B
a Winberg et al. [37].
b Vassaet et al. [38].
c Nakamura et al. [39].
d Georges et al. [40].

#### 3.1.2. *DAMD–PCR*

Five DAMD primers yielded 47 fragments, of which 43 were polymorphic (91.48%). The amplified fragments ranged from 0.2 to 3 kb with an average of 9.40 fragments per primer. HVR, M13, YNZ22 and PER1 exhibit highest level of polymorphism and the percentage of polymorphic bands was calculated as 100% for all these primers (Table 2; Fig. 2B). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.52 to 0.97 (Table 3).

#### 3.1.3. *Combined study of DAMD and ISSR*

Cumulative dataset was used to estimate the efficiency of both single primer based amplification reactions commonly regarded as SPAR to determine genetic variation at intra- and inter-specific levels. A total of 149 fragments were produced collectively, of which 113 fragments were polymorphic (75.83%) with an average polymorphic bands of 5.94 fragments per primer, and genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.65 to 0.93 (Table 3).

#### 3.1.4. *Population structure*

The percentage of polymorphic loci (P) after using combined data ranged from 22.82% to 44.30% (Table 4). Nei's gene diversity ($H_E$) values varied from 0.0831 to 0.1723 with a total genetic diversity ($H_T$) 0.2024 and Shannon's diversity values (*Hpop*) ranged from 0.1246 to 0.2541with a genetic diversity at inter-populations level ($D_{ST}$) 0.0715 and genetic diversity at intra-populations level ($H_S$) 0.1309 with a significant difference being found between populations. The genetic differentiation inter-populations level (*Gst*) values was 0.4053 and estimate of gene flow (N*m*) was 0.8085. AMOVA analysis of pairwise distances indicated high variation (68.88%) at intra-populations level, and moderate level of variation (31.12%) at inter-population level (Table 5).

#### 3.1.5. *Cluster/tree analysis*

Two major clusters were formed using UPGMA method of clustering (Fig. 3.) *viz.*, Cluster I and Cluster II. Cluster I could be sub-divided into four sub-clusters i.e., Sub-cluster IA, IB, IC and ID. Sub-cluster IA comprised 12 genotypes, out of which 6 genotypes belonged to Ri-Bhoi (SK-RB1 to SK-RB6), 4 to Kamrup-Metro (SK-KM1, SK-KM4, SK-KM5 and SK-KM6), and only one each to Sonitpur (SK-ST1) and Nagaon (SK-NG1) districts respectively. Sub-cluster IB consisted of 8 genotypes, of which 5 belonged to Nagoan (SK-NG2 to SK-NG6), 2 to Kamrup-Metro (SK-KM2 and SK-KM3) and only one to Sonitpur (SK-ST2) districts. Interestingly, sub-cluster IC comprised of 6 genotypes and all of them belonged to South Garo Hills. Sub-cluster ID comprised 7 genotypes, of which 3 were from Sonitpur (SK-ST3, SK-ST5 and SK-ST6) and the rest 4 belonged to West Garo Hills (SK-WG1 to SK-WG4). Cluster II represented only 2 genotypes and both belonged to West Garo Hills (SK-WG5 and SK-WG6).

## 4. Discussion

*J. curcas* as an important bioresource for biofuel exhibits high potential to support the development of a commercially viable biodiesel program. However, to ensure a sustainable

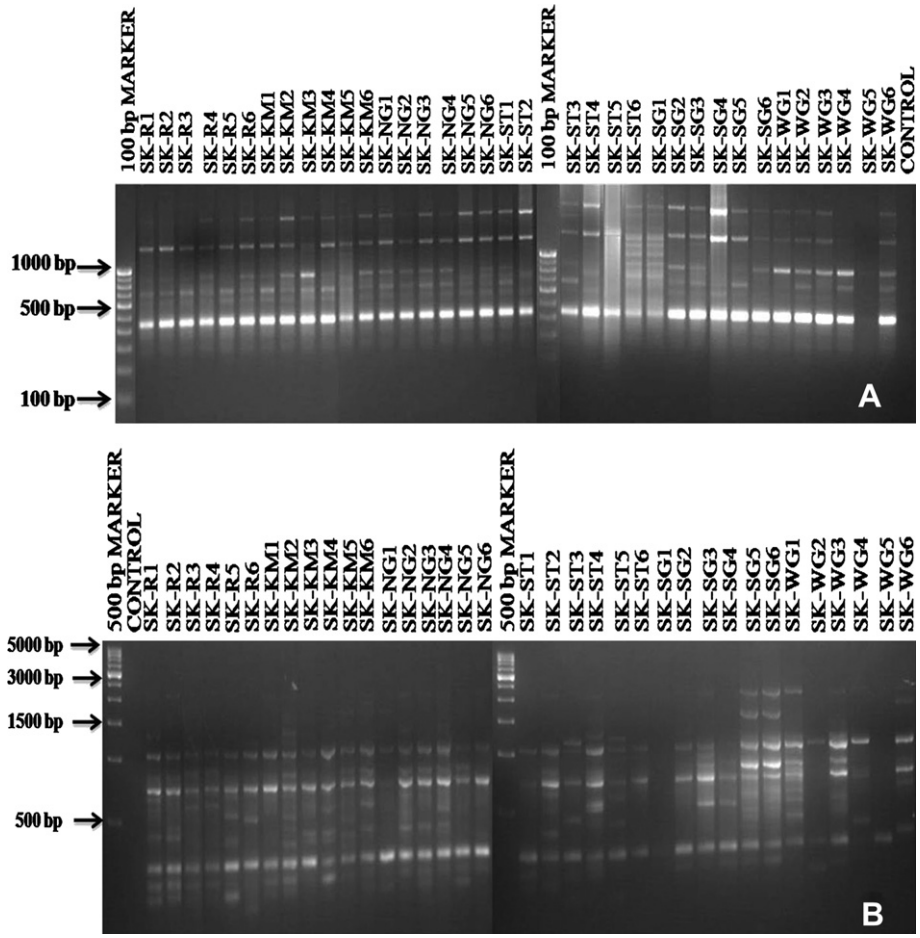


**Fig. 2 – Banding profile in *J.curacs* populations using ISSR primer A2 (A), and DAMD primer PER1 (B).**

**Table 3 – Comparison of SPAR methods (ISSR and DAMD), individually as well as collectively.**

| Sl No | Name of the SPAR Approach | No. of primer used | Total bands amplified | Average bands/ primer | Size of ampicons (range kb) | Total No. of polymorphic bands | Average no. of polymorphic bands/primer | Average % polymorphism | Distance range (Jaccard's Coefficient) | Average Distance |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ISSR | 14 | 102 | 7.28 | 0.2 – 3.0 | 70 | 5 | 68.62 | 0.73 – 0.95 | 0.84 |
| 2 | DAMD | 5 | 47 | 9.4 | 0.2 – 2.5 | 43 | 8.6 | 91.48 | 0.52 – 0.97 | 0.74 |
| 3 | ISSR + DAMD | 19 | 149 | 7.84 | 0.22 – 3.0 | 113 | 5.94 | 75.83 | 0.65 – 0.93 | 0.79 |

**Table 4 – Analysis of divergence of genetic variation in six populatios of *J. curcas*.**

| Population | Na ± SD | Ne ± SD | $H_E$ ± SD | $H_{pop}$ ± SD | P (%) | Number of polymorphic loci |
|---|---|---|---|---|---|---|
| Pop1 | 1.2416 ± 0.4295 | 1.1441 ± 0.2863 | 0.0865 ± 0.1610 | 0.1302 ± 0.2372 | 24.16 | 36 |
| Pop2 | 1.4295 ± 0.4967 | 1.2721 ± 0.3527 | 0.1607 ± 0.1955 | 0.2394 ± 0.2848 | 34.23 | 51 |
| Pop3 | 1.3423 ± 0.4761 | 1.2108 ± 0.3238 | 0.1260 ± 0.1831 | 0.1886 ± 0.2687 | 42.95 | 64 |
| Pop4 | 1.3221 ± 0.4689 | 1.2059 ± 0.3275 | 0.1216 ± 0.1841 | 0.1807 ± 0.2690 | 32.21 | 48 |
| Pop5 | 1.2282 ± 0.4211 | 1.4403 ± 0.3725 | 0.0831 ± 0.1606 | 0.1246 ± 0.2357 | 22.82 | 34 |
| Pop6 | 1.4430 ± 0.4984 | 1.2980 ± 0.3725 | 0.1723 ± 0.2033 | 0.2541 ± 0.2937 | 44.30 | 66 |
| Mean | 1.7517 ± 0.4335 | 1.3562 ± 0.3345 | 0.2202 ± 0.763 | 0.3420 ± 0.2476 | 29.64 | 49.83 |

$H_E$ = Nei's (1973) gene diversity; $H_{pop}$ = Shannon's Information index (Lewontin 1972); Na = Observed number of alleles; Ne = Effective number of alleles (Kimura and Crow 1964); P = percentage of polymorphic loci (Lynch and Milligan 1994); SD = Standard deviation.

**Table 5 – Results of analysis of molecular variance (AMOVA) of SPAR methods for six populations of *J. curcas*.**

| Source of variation | Degrees of freedom | Sum of squares | Mean squared deviation | Variance component | Percentage of variation |
|---|---|---|---|---|---|
| Inter-population level | 5 | 207.389 | 41.477 | 5.05 | 31.12 |
| Intra-population level | 30 | 335.333 | 11.177 | 11.17 | 68.88 |
| Total | 35 | 542.722 | 52.654 | 16.227 | 100 |

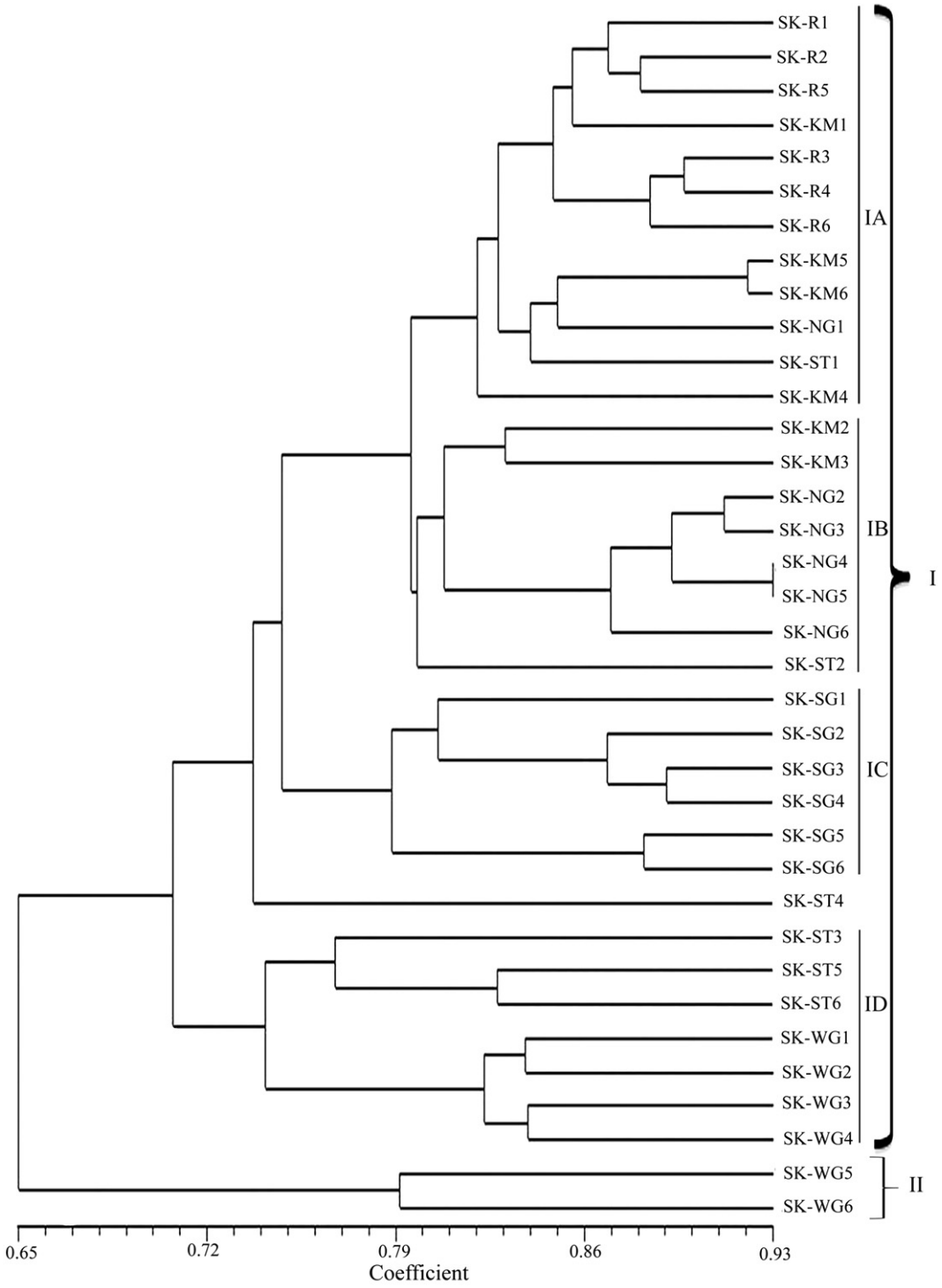


**Fig. 3 – UPGMA clustering of *J. curcas* population.**

utilization of this important bioresource it is necessary to collect and evaluate germplasm in such a way that the maximum diversity in terms of genetic and molecular levels should be included. Since it is a cross-pollinated crop, genetic improvement and breeding programmes should involve discrete populations rather than individual breeding lines [1]. Therefore, tools suitable for the assessment of polymorphism at genomic level are very essential to readily identify the genetically distinct populations which can be selected for performance, evaluation and breeding programmes. *J. curcas* exhibits enormous phenotypic diversity and some of the variations may have a genetic basis too. SPAR approach has been used to analyze genetic diversity at intra- as well as inter-specific levels in several tree species [15,29–32]. Therefore, the present work has been initiated to compare and utilize SPARs as an effective genetic marker for assessing the genetic diversity in *J. curcas*.

In the present investigation, the cumulative dataset (ISSR and DAMD markers) revealed 75.83% polymorphism. The presence of distinct polymorphism in a given population is often due to the existence of genetic variants represented by the number of alleles at a locus and their frequency of distribution in a population. Till date very few studies have been carried out to understand the genetic diversity among the populations of *J. curcas* from India [13–18]. In the previous genetic analysis studies reported so far on *J. curcas*, the materials mostly represented areas other than those of Northeast India. The present study, which includes wild germplasm of Northeast India reveal unique polymorphism in *J. curcas* populations. The moderate level of variation at inter-population level may be attributed to a small amount of introductions/races of the species in the area and its further distribution through vegetative propagation and anthropogenic activities as reported in earlier studies [13]. The dendogram constructed for cumulative dataset (ISSR and DAMD) revealed that most of the genotypes clustered together except WG-5 and WG-6; such evident grouping may be attributed to the possible heterozygosity among the genotypes and reflected at DNA level by cumulative dataset. Earlier studies [13,33] on genetic diversity analysis were based on geographical isolation and environmental- influenced genetic differentiations in *J. curcas* and allied species. However, clustering pattern in present study did not clearly show any geographical isolation and/or ecological differentiations. Hence, such observations may reflect that geographical differentiation of *J. curcas* in Northeast India is not pronounced.

Some of the interesting facts revealed through present investigation are in partial agreement with the study of Gupta et al. [18]. The positive aspects describe that microsatellites are more frequent in *Jatropha* which contains di-, tri- and penta-nucleotides, particularly repeat nucleotides of poly (GA) and poly (AG) primers. The primers that were based on these repeats showed significant polymorphism also in our study. However, in contrast to the study of Gupta et al. [18], the present observation showed noteworthy amplification with poly (TG), poly (CA), poly (GT) and especially (ACC) n showed 100% polymorphism which was not reported in their work. This result clearly shows Mendelian inheritance in North-eastern genotypes of *J. curcas* with precise polymorphism and incises the non-availability of such repeats in the genome of *J. curcas* as described by Gupta et al. [18]. The absence of poly (AT) di-nucleotide repeats that are thought to be the most abundant motifs in plant species are characteristically absent in the present study as well [34,35].

In the present investigation, most of the DAMD primers showed 100% polymorphism with absence of (AT) repeats draws the favor of underlying broad genetic base of *J. curcas* genome. These kinds of results revealed the information that genomes of genotypes of the same species may vary in sequence as a result of different evolutionary process at intra- and inter-population levels [36].

The present study based on SPAR methods revealed high genetic diversity at intra-population level but moderate genetic diversity at inter-population level of *J. curacs* collected from Northeast India. Identification, collection and maintenance of such genetically diverse wild germplasm from Northeast India would be of great importance in economic improvement of this species. Our work indicated that ISSR and DAMD markers could be used for estimation of genetic relationships, which ultimately would be helpful in characterization of various biofuel genetic resources.

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## Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.biombioe.2011.04.025.

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