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Assessment of genetic diversity in *Shorea robusta*: an economically important tropical tree species

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ABSTRACT

The present investigation reports an elucidation of genetic diversity among four-populations of most economically and ecologically important tree species, sal (*Shorea robusta* Gaertn.) for the first time in India, using ISSR markers. A total of one-hundred individual *S. robusta* trees were sampled from four different populations, considering twenty-five individuals from each population. In total, twenty-ISSR primers were screened with *S. robusta* DNA, and out of twenty, sixteen-primer produced reproducible amplicons. Sixteen selected ISSR markers were amplified a total of 118 alleles and the total number of amplicons for individual primers ranged from 5 to 12, with a mean of 7.37 alleles per primer, of which 74 were polymorphic with an average of 4.62 alleles per primer. The ISSR primer $(GA)_8YG$ yielded highest number of alleles (12) and primers $(CA)_8RG$ and $(CT)_8G$ yielded lowest number of alleles (5), with an average alleles size between 200-3500bp. The percentage of polymorphic alleles ranged from 40 [$(CA)_8RG$] to 83.33 [$(AC)_8C$]. A dendrogram based on UPGMA analysis grouped the four populations into two major clusters, having Keonjhar population into first cluster and rest three populations into second cluster. It was notable that the second major cluster was further divided into three-separate sub-clusters, representing population from each three locations. Analysis of molecular variance (AMOVA) revealed that the majority of genetic variation exists within populations, compared to the variation that exists among the populations. The present study is a clear-cut indication that inter- and intra-population genetic variation exists in *S. robusta* and ISSRs appears to be an efficient marker system in quantifying genetic variability in different populations of *S. robusta*.

1. INTRODUCTION

Trees are major components of forests/ wild and essential for maintaining health of several ecosystems. Sal (*Shorea robusta* Gaertn.) is a tropical tree species belonging to the Dipterocarpaceae which consists of three sub-families, 17 genera and 511 spp. [1]. It is a hermaphrodite species which attains a height up to 30-35m and trunk (girth) diameter of up to 2.0-2.5m. The species is naturally found in Bhutan, Bangladesh, Nepal and India, and covers more than 12 million hectares of forest area [2], and is economically and ecologically important in that region.

Champion and Seth [3] have demarcated the distribution of *S. robusta* forests ranging from Uttarakhand in the north up to Andhra Pradesh in the south and Tripura in the east; covering Himachal Pradesh, Haryana, Uttar Pradesh, Bhihar, West Bengal, Odisha, Madya Pradesh, Chattisgarh, Maharastra, Jharkhand, Sikkim, Assam and Meghalaya. In wetter areas, it is evergreen; in drier areas, it is dry-deciduous, shedding most of the leaves in between February to April, leafing out again in April and May. Other Shorea species are insect-pollinated and wind is the exclusive pollen vector in *S. robusta* [4]. Seeds of *S. robusta* are ovoid in shape (~8mm in diameter), weighing up to 2g, with two shorter and three longer wings [5] Seed dispersal is wind-driven and seeds of *S. robusta* are recalcitrant, viability is lost within a week after falling to the ground [2].

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Though the plants produce flowers and fruits luxuriantly in the plains and foothills, its overall natural regeneration is very poor [6,7]. Further, it exhibits large scale mortality or die-back at the seedling stage due to drought [8]. Past records provide evidence of larger distribution of *S. robusta* forests in the northern and eastern parts of India [9], and their eventual clearance for expanding agriculture, human settlement etc. [10]. During the past decades, there was massive deforestation to use the wood as railway sleepers, ship-building and other load bearing purposes etc., resulting in the decreasing of natural populations in the wild. In addition, Sal forests yield non-timber forest products, including fodder, seed for oil, tannin and gum from bark [11] and leaves for plate making [12], hence it is one of the economically important forest tree species.

The conservation and management of plant species, in addition to ecological information, requires a sound understanding of underlying genetic processes as well as variation within and among populations [13]. Genetic diversity and natural distribution pattern are very important for the introduction and conservation of forest plant species in general and *S. robusta* in particular. In order to obtain such information, highly sensitive techniques are required, which should be simple, informative and cost efficient. In recent past, isozyme profiling has been used to assess genetic diversity in *S. robusta* populations of Nepal [14]. However, Biochemical markers such as isozyme and protein patterns, are influenced by the developmental stage of the plant as well as environmental factors; moreover, they offer limited polymorphism and often do not allow discrimination among closely related cultivars [15] and species. Pandey and Geburek [16] have investigated the genetic diversity and structure at four-microsatellites of 15 populations of *S. robusta* comprising continuous-peripheral and disjunct-peripheral populations in Nepal. In another study, the same authors have conducted the genetic diversity analysis between adults and juveniles in a semi-isolated natural population of *S. robusta* in Nepal [17]. However, to date no such genetic diversity assessment investigations exists for the *S. robusta* natural tree populations in India.

In order to identify the level of diversity, DNA based molecular marker techniques have become indispensable. Extensive studies have shown that the DNA markers provide highly efficient and informative way to characterize the diversity at population level [18-22]. Widely used PCR-based marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSRs) or microsatellites [23,24]. However, in some instances low reproducibility of RAPD [25,26] and high cost of AFLP, and the need to develop species-specific primers for SSR analysis are major limitations, all of which can be overcome by the inter simple sequence repeats (ISSR) markers [27, 28]. ISSR primers of repeat sequences (microsatellites) are reported to be dispersed throughout genomes anchored either at 5' or 3' end with one or few specific nucleotides and amplify the sequences between the two microsatellite loci [29]. Longer primers (16-25 mer) permit the use of high annealing temperatures (45-60°C), which is probably the reason for the improved reproducibility and reliability of ISSR compared to RAPD where 10-mer primers are used [30].

Currently, no DNA fingerprinting investigation had been carried out to assess the genetic diversity of *S. robusta* populations in India using molecular markers. Therefore, this pioneering investigation aimed at determining the genetic variability assessment within and among different natural *S. robusta* tree populations exists in tropical moist deciduous forest regions in Odisha, India using ISSRs. The outcome of the study is highly useful in designing future conservation strategies for this important dominant tree species.

2. MATERIALS AND METHODS

2.1 Study sites and plant sampling

Fresh and young leaf samples were collected from four different natural tree populations of *S. robusta* present in different regions of Odisha, India i.e. Kadukaman of Kalahandi South Forest Division; Athamalik of Hatidara Forest Division; Benmunda and Bandhori of Keonjhar Forest Division; Tamana and Rajin of Khorda Forest Division. Each population was positioned by a Global Positioning System (GPS), and the location details are depicted in Figure-1 and Table-1. In each population, individual's trees were randomly selected for sample collection. In total 100-individual leaf samples from four different populations were collected for the DNA fingerprinting studies. The locations of the populations, climatic conditions and forest type were recorded for each of the populations (Table 1). Morphological characteristics like individual tree height, canopy length and girth diameter were recorded for each of the trees from all four experimental locations.

Table 1: Description of *S. robusta* samples collected from different locations in Odisha, India.

Population	Population code	Type of forest	Av. annual rainfall (mm)	GPS reading			Number of samples
				Latitude	Longitude	Altitude/elevation	
Kadukaman, Kalahandi South Forest Division	KHD	Reserve forest	1378.20	19° 36'.065'N	083° 08.726' E	753m	25
Athamalik, Hatidara Forest Division	ATH	Reserve forest	1421.00	20° 44.182' N	084° 40.589' E	343m	25
Benmunda and Bandhori, Keonjhar Forest Division	KJR	Reserve forest	1534.50	21° 13'.325' N	085° 30.518' E	220 m	25
Tamana and Rajin Khurda Forest Division	TMN	Reserve forest	1449.10	19° 53'.349' N	084° 59.769' E	520 m	25

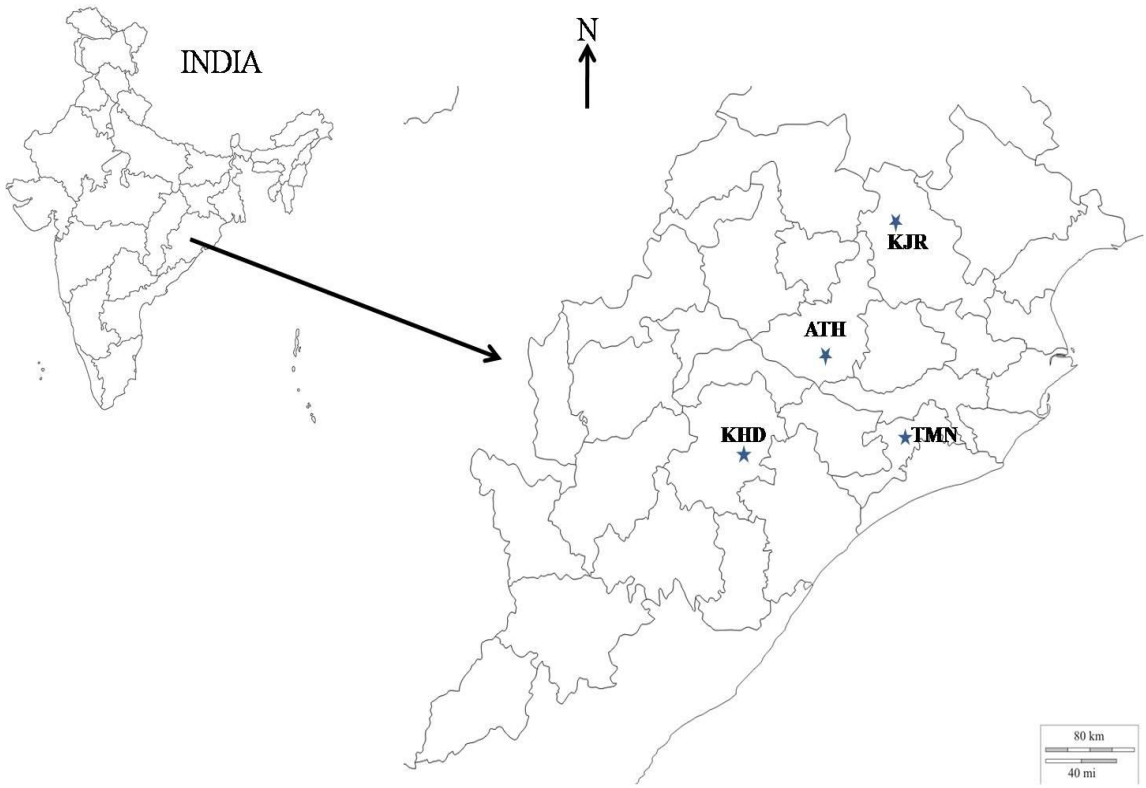

Fig. 1: Map of India showing the sampled populations of *S. robusta*. KHD, Kalahandi; ATH, Athamalik; KJR, Keonjhar and TMN, Tamana populations.

2.2 DNA extraction

One gram of leaf material was ground using liquid nitrogen. Genomic DNA was isolated from approximately 100mg of finely ground powder using DNeasy Plant Mini Kit (Qiagen, Cat. No 69104) as per the manufacturers recommendations.

The quality of genomic DNA was examined by 0.8% agarose (w/v) gel electrophoresis and quantified spectrophotometrically at A260 and A280 nm. Each sample was diluted to 50 ng/µl in TE buffer and stored at -20°C, until use.

2.3 ISSR primer screening

Primers were selected on the basis of their ability to detect distinct, clearly resolved and polymorphic amplified products after screening with *S. robusta* DNA, for further analysis. The primers that gave either negative or weak/complex allele patterns were discarded, while those giving reproducible patterns were adopted for further detailed characterization.

In total, 20-ISSR primers (IDT, India) were screened, and based on the number and reproducibility of the polymorphic fragments, 16-primers were selected for further analysis. The details of primers used are presented in Table-2.

2.4 ISSR-PCR amplification

PCR amplification reaction for ISSR analysis were conducted in a total volume of 25 µl reaction mixture in a 0.2 ml PCR tube which consisted of 50ng of template DNA, 1×Taq buffer-A (10 mM Tris-Cl [pH 9.0]), 50 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin, 100µM of each dNTPs (dATP, dTTP, dCTP and dGTP), 20µM primer, 5Unit/µl Taq DNA polymerase (GeNei, India). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) programed for a preliminary 2 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 30s, annealing at required temperature (depending on Tm of the ISSR primer) for 1 min, extension at 72°C for 2 min, and finally extension at 72°C for 7 min.

2.5 Agarose gel electrophoresis and imaging

Separation of amplified DNA fragments were performed by electrophoresis in 1.8% (w/v) agarose gel along with a molecular weight marker (500bp) for comparison, pre-stained with ethidium bromide and visualized under UV light and captured the image by ChemiDoc gel imaging system (Bio-Rad) for further data analysis.

Table 2: List of ISSR primers, total number of amplified fragments, number of mono- and poly-morphic fragments, and percentage of polymorphism generated in the *S. robusta*.

S. No.	Primer sequence (5'-3')	Length of amplified alleles (bp)	T_m	Total no. of alleles	No. of monomorphic alleles	No. of polymorphic alleles	Polymorphism (%)
1	CACACACACACACACARG	200-1300	52.7°C	5	3	2	40
2	TGGACACACACACACAC	300-1600	47.4°C	8	4	4	50
3	ACACACACACACACACC	450-1500	54.8°C	6	1	5	83.33
4	CACACACACACACACAG	300-1700	53.3°C	9	4	5	55.55
5	GAGAGAGAGAGAGAGAYT	350-1500	52.1°C	6	2	4	66.66
6	GAGAGAGAGAGAGAGAYG	250-1600	52.9°C	12	5	7	58.33
7	AGAGAGAGAGAGAGAGYT	400-2000	50.0°C	9	3	6	66.66
8	AGGGCTGGAGGAGGGC	400-2500	49.8°C	9	3	6	66.66
9	AGAGAGAGAGAGAGAGC	200-3500	52.8°C	6	2	4	66.66
10	GAGGGTGGAGGATCT	450-2400	49.1°C	8	3	5	62.50
11	CTCTCTCTCTCTCTCTG	300-1600	46.8°C	5	1	4	80
12	AGAGAGAGAGAGAGAGT	450-1400	52.4°C	6	2	4	66.66
13	ACGGTGTGTGTGTGTGT	450-1550	54.5°C	9	2	7	77.77
14	GAAGAAGAAGAAGAAGAA	350-1500	43.2°C	7	3	4	57.14
15	ACAGACAGACAGACAG	400-1550	47.4°C	6	2	4	66.66
16	CAGCGACAAG	450-3000	33.5°C	7	4	3	42.85
	Total			118	44	74	1007.43
	Average per primer			7.375	2.75	4.625	62.96

2.6 Data scoring and statistical analysis

The experiments were repeated twice for each of the ISSR primer used, and the clear, reproducible, and scorable allele classes were scored using Quantity One software (Bio-Rad) and were converted into binary data matrices on the basis of their presence (1) or absence (0) in the gel. All the alleles were scored to avoid over/under estimate the diversity [31]. Diffused alleles or alleles revealing ambiguity in scoring were considered as missing data and designated as '9'. Genetic similarity between pairs was calculated according to Jaccard's similarity coefficient (UPGMA), and a dendrogram was generated by using NTSYS-pc version 2.1software. Analysis of molecular variance (AMOVA) estimated to know the % polymorphism exists within and among populations of sal. Principal component analysis (PCA) based on Jaccard's similarity was used to estimate the actual number of groups that may be obtained by cluster analysis. Diversity values were calculated according to *Nei's* unbiased statistics using POPGENE.

3. RESULTS

3.1 ISSR primer polymorphism

Sampling (consisting 100-adult trees) was carried out in a sal dominated natural moist deciduous forest regions exist within Odisha state, India (Table 1). Out of 20-ISSR primers screened, 16-ISSR primers produced clear, scorable and reproducible DNA allele classes. The 16-ISSR markers amplified a total of 118 alleles and the total number of scored alleles varied from 5 to 12 for different primers, with a mean of 7.37 alleles per primer (Table 2), of which 74 were polymorphic with an average of 4.625 polymorphic alleles per primer. ISSR primer $(GA)_8YG$ yielded the highest number of alleles s (12) and primers $(CA)_8RG$ and $(CT)_8G$ yielded lowest number of alleles (5) with average allele size between 200-3500bp. The percentage of polymorphism for individual primers ranged from 40% [$(CA)_8RG$] to 83.33%[$(AC)_8C$] (Table 2). The ISSR fingerprinting profile obtained by using the primer 5'-AGGGCTGGAGGAGGGC-3' in ATH population are depicted as Figure-2.

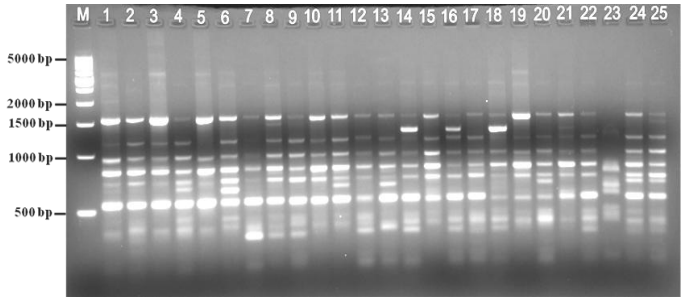

Fig. 2: ISSR fingerprinting profile of 25-individuals of *S. robusta* using primer- 5'-AGGGCTGGAGGAGGGC-3' and 'M' is a molecular size marker (500bp).

3.2 Genetic variation within and among population

The percentage of polymorphic loci recorded highest for the KJR (86.54%) and lowest for the ATH (70.51%) populations, with an average value of 79.32% (Table 5). The effective number of alleles (Ae) showed variation in the rage of 1.359 (KHD) to 1.404 (KJR and TMN) with a mean of 1.386, and recorded 1.710 at species level. Nei's gene diversity (He) values ranged from 0.219 (KHD) to 0.251 (TMN) with an average of 0.251 and at species level recorded 0.394. Shannon's information index (Io) observed highest in TMN (0.389) and lowest in ATH (0.333) population with an average value of 0.360, and at species level recorded 0.573. Further, among the four populations studied, ATH showed lowest level of variability and KJR (0.382), TMN (0.389) exhibited highest variability, respectively. The gene flow (Nm) estimated using ISSRs was low (0.3649) per generation (Table-5). Analysis of molecular variance (AMOVA) showed highly significant ($P<0.001$) genetic variability among populations and revealed a higher proportion of genetic variability within populations (54.06%) compared to among population genetic variability (45.94%) (Table-3).

Table 3: Analysis of molecular variance (AMOVA) for 100-individuals in four populations of *S. robusta*.

Source of variation	d.f.	SSD	MSD	Est. Var.	TV (%)	*p*-value
Among population	3	1267.28	422.42	16.13	45.94	<0.001
Within population	196	1822.96	18.98	18.98	54.06	<0.001
Total	199	3090.24	441.40	35.11		

df, degree of freedom; SSD, sum of squared deviation; MSD, mean sum of squared deviation; Est. var., estimated variance; TV %, total variance percentage*p*-value, probability value.

3.3 Genetic relationship between populations

The pair wise genetic identity value among the populations ranged from 0.6915 between KHD and KJR to 0.7517 between KHD and ATH, with a mean of 0.7216 (Table-4). Further, genetic distance was higher for TMN and KJR populations (0.3520) and lowest for KJR and KHD populations (0.0649) (Table-4). The similarity matrix representing Jaccard's similarity coefficient was used for clustering four-populations, adopting UPGMA algorithms similarity matrix. The clustering of different populations based on Jaccard's coefficients presented in Figure-3, and which grouped the populations into two major clusters. Cluster-I represents the KJR sal population alone. Cluster-II again divided into two sub-clusters; these are sub-cluster-a and sub-cluster-b. Sub cluster-a represents the TMN population alone and the sub-cluster-b represents the ATH and KHD populations, respectively (Figure 3). The KJR population is genetically the most distant from all others, followed by TMN. The clustering pattern under study revealed considerable genetic variability within populations. The grouping of sal populations through principal coordinate analysis (PCA) also resulted in similar trends as observed through three-dimensional Principal Co-ordinate Analysis (Figure-4).

Table 4: Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) between the populations of *S. robusta* by ISSR.

Populations	KHD	ATH	KJR	TMN
KHD	***	0.7517	0.6915	0.7442
ATH	0.2855	***	0.7232	0.7243
KJR	0.0649	0.3241	***	0.7033
TMN	0.2954	0.3226	0.3520	***

Table 5: Genetic variability within populations of *S. robusta* detected by ISSR analysis.

Population	A_O	A_e	H_o	I_o	P (%)	Nm
KHD	1.750 (0.43)	1.359 (0.35)	0.219 (0.18)	0.339 (0.25)	75.00	-
ATH	1.705 (0.45)	1.380 (0.38)	0.220 (0.19)	0.333 (0.27)	70.51	-
KJR	1.865 (0.34)	1.404 (0.34)	0.246 (0.17)	0.382 (0.23)	86.54	-
TMN	1.852 (0.35)	1.404 (0.32)	0.251 (0.16)	0.389 (0.22)	85.26	-
Mean	1.793	1.386	0.234	0.360	79.32	-
At species level	1.980	1.710	0.394	0.573	98.08	0.3649
SD	0.13	0.28	0.12	0.15		

A_O-observed number of alleles, A_e-the effective number of alleles, H_o-Nei's gene diversity, I_o-Shannons's information index, P-percentage polymorphism, Nm-Gene flow.

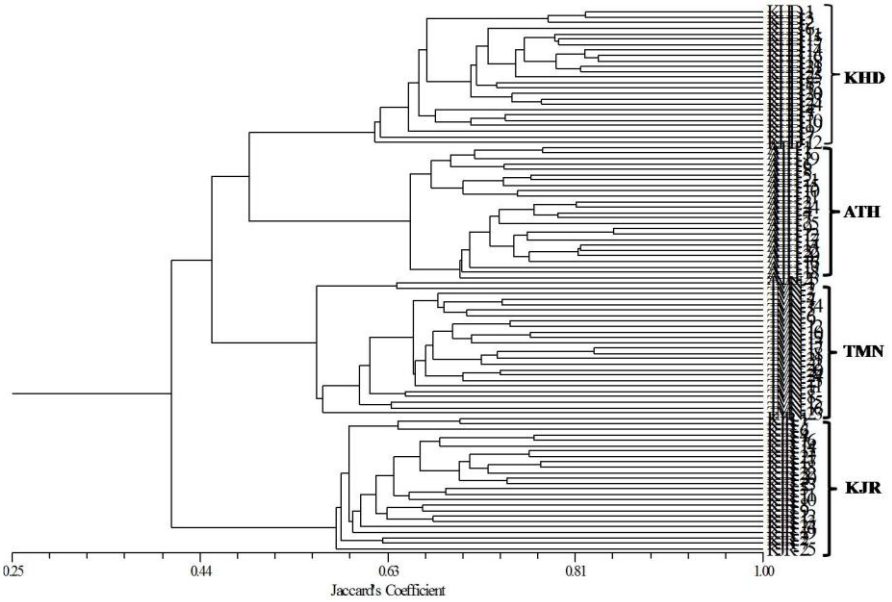

Fig. 3: UPGMA-based dendrogram representing genetic relationships among four-populations of *S. robusta* based on Jaccard's similarity coefficients.

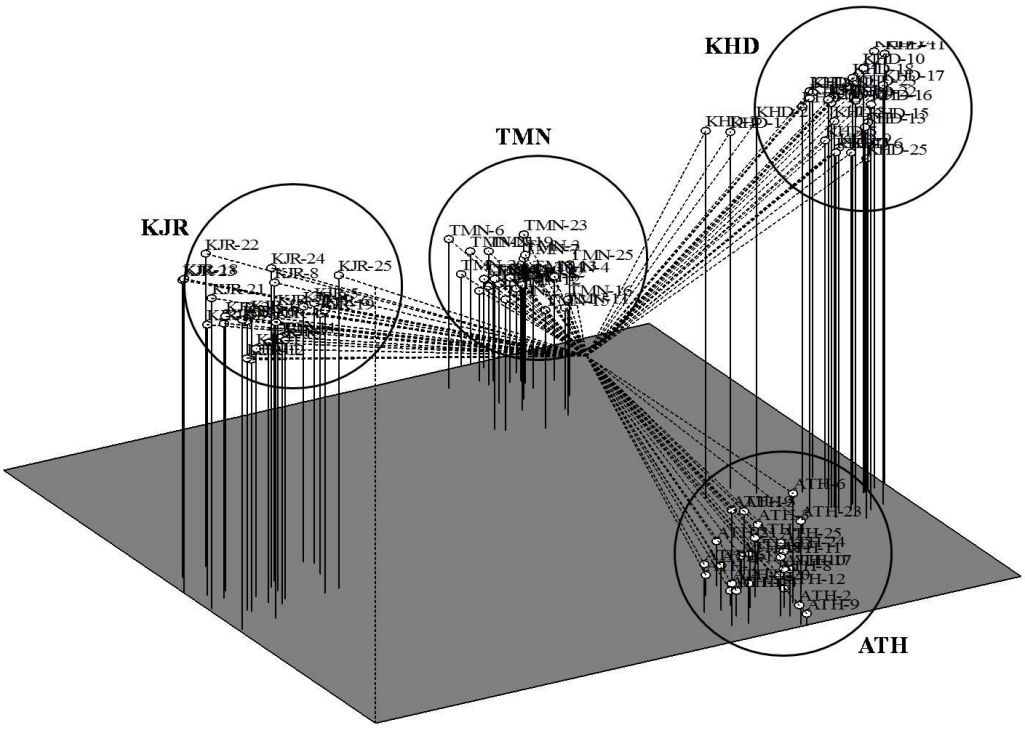

Fig. 4: 3D plot shows distribution of 100- *S. robusta* tree individuals from four different populations by principal coordinate analysis.

4. DISCUSSION

The present study reports genetic variability assessment in different populations of tropical moist deciduous forest tree species, *S. robusta* for the first time in India. In order to analyze genetic diversity, 100-individual tree-DNA samples were subjected to 20-ISSR markers, and based on reproducible, scorable allele classes, 16-primers were considered for further analysis. The tested ISSR primers were allowed the discovery of reasonably high level of polymorphism (average 63%) and are a reflection of presence of a wide range of genetic variation among the individuals examined. The present findings seem to be relatively similar when compared to the reports of previous ISSR studies on different tree species [32-34]. Our study demonstrates for the first time that the ISSR-PCR a suitable method for detection of genetic variability in sal. The rates of evolution in different parts of the genome are extremely variable, allowing molecular data to be applicable at any taxonomic level. Further, an outstanding advantage of a molecular approach is the immense amount of potential data it provides [21, 32, 35, 36] as evidenced by the current investigation.

The genetic variability (Nei's genetic diversity,) at population levels were high (0.234) in the present study, as compared to similar studies conducted in different plant species i.e. in *Roscoea procera* Wall.- 0.166 [20], in *Astragalus sericeocanus* Gontsch.- 0.112 [22]. However, the level of genetic diversity in the present study was more or less similar to *Saraca asoca* [19]. Shannon's information index (*I*o) mean value (0.360) observed in this study was higher than the similar recent studies conducted on different plant species such as in *Roscoea procera* Wall., 0.263 [20]; in *Astragalus sericeocanus* Gontsch., 0.173 [22] using ISSRs and in *Saraca asoca* (Roxb) De Wilde, 0.152 [19] using RAPDs, confirming its (pollen reception from xeno and geitono, and cryptic self-incompatibility) out crossing nature. However, it is argued that long-lived species like sal tree should strongly favor out crossing [37].

The analysis of molecular variance (AMOVA) in the current study revealed a significantly (<0.001) high variance (54.06%) within the populations compared to variance that exists among the populations (45.94%) and our results are in agreement with the trend observed in perennials and long-lived tree species [19, 20]. The gene flow (Nm) pertains to gene movement across intra- and inter-population levels and Nm largely shows impact on genetic differentiation in populations [34]. The low level of gene flow and high level of inter population differentiation in the present study could be a reflection of limited seed and pollen dispersal and inbreeding nature of *S. robusta* populations. Further, *S. robusta* being a long-lived tree species has maintained a high genetic diversity. The genetic differentiation among the populations could be the result of an adaptation to environmental gradients. In particular, *S. robusta* is distributed across different eco-geographic conditions with respect to soil composition, altitude etc. which could drive the acquisition of total adaptation. In addition, the genetic diversity of a plant species can be governed by different factors such as distribution range, life form, breeding system, seed dispersal mechanism mode of reproduction and successional status [38,39]. Of these, breeding system appears to be the most important factor, followed by life form, seed

dispersal and successional status [40] It has been reported that a species, which has a long life, high frequency of gene flow and more number of seed, tends to possess high genetic diversity [41]. However, among populations variance value is higher side compared to recent reports on different plant species [19,20], indicating the necessity of enhancing the gene flow among populations by cross-re-introduction of individuals and implementation of *in situ* conservation of existing populations.

Both UPGMA-phenogram as well as PCA displayed similar grouping of populations. The PCA results corresponded well with the grouping of populations based on cluster analysis. The dendrogram (Jaccard's similarity coefficient) showed highest genetic similarity between populations KHD, ATH and TMN compared to KJR population. The higher genetic similarity indicates that higher probability of origin of all these populations from the same ancestral source, eventually distributed to different locations and also geographically close locations. However, it is true that the higher genetic diversity of the *S. robusta* populations in Odisha, India is largely due to a bigger population size and distribution, and similar reports were observed for different tree populations i.e. in *Roscoea procera* [20] and in *Saraca asoca* [19].

5. CONCLUSION

In conclusion, the molecular analysis based on an amplification signal using ISSR markers is sufficiently informative and powerful to authentically elucidate genetic variability in *S. robusta*. The ISSR markers reported in the present study will facilitate the understanding of genetic variability, inter-population gene flow and evolutionary relationships in *S. robusta.* The Keonjhar *S. robusta* population harbor highest genetic diversity thereby deserving higher priority for *in situ* conservation. Overall high genetic diversity, limited gene flow and genetic differentiation indicates the need for implementing *in situ* and *ex situ* conservation of studied *S. robusta* populations.

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