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Original Research article

Genetic Diversity Analysis of *Diospyros melanoxylon* Roxb. (Kendu) Population Growing in Jharkhand, India Using ISSR Markers

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Abstract: *Diospyros melanoxylon* Roxb. (family-Ebanaceae), commonly known as Kendu or Tendu, is an economically important tree species of the Indian forests. Its leaves are used for making 'bidi'. Besides contributing an important role in the economy of rural people, its leaves, barks and fruits have been used since ancient times in Indian traditional medicinal system- 'Ayurveda' to cure a variety of diseases. The aim of the present study was to determine the nature and extent of genetic diversity of *D. melanoxylon* Roxb. germplasm resources, using ISSR markers. For assessment of population diversity status, a total of 48 germplasm growing in 8 districts of Jharkhand were collected. The ISSR fingerprints detected 85.0% polymorphism among 120 genetic loci amplified. Dendrogram based on binary data matrix resulted into 5 clusters. The calculated mean value for Shannon's index (I), Nei's genetic diversity (*h*), observed numbers of allele (*na*), effective numbers of alleles (*ne*) was 0.4641, 0.3093, 1.8917 and 1.5302, respectively. The gene flow was estimatedto be 1.6533, indicative of a high amount of genetic exchange within the species. Apportionment of genetic variability by AMOVA revealed that most of the diversity (86.6%) was distributed between individuals within a population followed by among region (12.29%) and between populations within regions (1.11%) respectively. The ISSR markers, thus utilised, proved to be very useful in deciphering the genetic diversity structure of naturally growing tree populations. The results obtained are very informative with regard to not only genetic diversity status but also from the point of view of utilization.
Key words: Genetic diversity, ISSR, Kendu, Nei's gene diversity, polymorphism

Introduction

Diospyros melanoxylon Roxb. (family-Ebanaceae), commonly known as Kendu or Tendu, is an economically important tree species of the Indian forests. Its leaves are used for making 'bidi'. Besides being a source of earning for rural people, its leaves, barks and fruits has been used since time immemorial in traditional 'Ayurvedic system' to cure a variety of diseases including diarrhoea, cholera, dysentery, intermittent fevers, cough, cramps, pneumonia, etc. (Kantamreddi and Wright 2008).

Deciphering the genetic diversity of a tree species is fundamental to effective conservation strategies and subsequently devising the plans for improvement of the species. In other words, the evaluation of genetic diversity of a germplasm resources is a prerequisite to the effective management of genetic resources, designing conservation programmes and breeding strategies, monitoring genetic erosion and development of 'core collection' for future reintroduction of the species. The estimation of disturbance and loss of diversity has become one of the most intriguing puzzle to be solved for molecular biologist, ecologist as well as natural resource managers in the last three-four decades (Oliver 1981; Miller 1982; Rykial 1985; Petraitis *et al.* 1989;

Ehrlich 1991; Peltzer *et al.* 2000). The major cause of species loss and biodiversity extinction has been attributed to habitat destruction (Pimm and Raven 2000; Koh *et al.* 2004).

PCR based marker technologies developed during late 20th century allowed the unscrambling of the extent of the genetic variation in an unparalleled way through better coverage of the genome. Molecular markers characterise a sample of plant genome which is used to understand relationship between entire genome within a set of population. ISSR fingerprinting is one such promising marker system developed by Zietkiewicz *et al.*, (1994) and Kantety *et al.*, (1995). In the present study ISSR markers have been utilised to investigate the level of genetic variation of different populations of *D. melanoxylon* at three hierarchical levels: (i) within population; (ii) among populations within regions; and (iii) among populations from different regions.

thus collected were used for the DNA extraction following the protocol as prescribed by Kumar *et al.*, (2014). The DNA thus obtained for all the 48 genotypes were used for ISSR-PCR analysis. Different concentrations of genomic DNA, $MgCl_2$ and *Taq* DNA polymerase, were tested in various combinations to ascertain the most suitable conditions for obtaining good and analysable amplification products. Among the constituents standardised, 50 ng genomic DNA, 2.0 mM $MgCl_2$ concentration and 0.75 unit *Taq* DNA polymerase (Xcelris, India), 2% formamide besides 2.5 µl of 10x assay buffer, 0.24 mM dNTPs and 5 µm primer in the reaction mixture were found to be most suitable for obtaining optimum and stable results.

A total of 50 SSR primers were initially tested. Out of these, 18 primers were found to produce good analyzable amplification products. These 18 ISSR primers were eventually used to amplify DNA from 48 genotypes.

Table 1. Collection of *D. melanoxylon* germplasm resources from Jharkhand and used in the present study

Region	District	Geographical Co-ordinates		Altitude (mts.)	Sample collection area (Forest Type)	Population (Pop) number	Number of genotypes	Codes used in the dendrogram
		Latitude	Longitude					
North Chotanagpur	Hazaribagh	23° 59'	85° 22'	610	Dense	Pop1	6	1-6
	Chatra	24° 43'	84° 20'	613	Dense	Pop2	6	7-12
	Giridih	24° 11'	86° 18'	289	Open	Pop3	6	13-18
	Dhanbad	23° 47'	86° 25'	227	Open	Pop4	6	19-24
	Bokaro	23° 40'	86° 9'	210	Open	Pop5	6	25-30
Palamu	Palamu	24° 12'	84° 18'	215	Open	Pop6	6	31-36
South Chotanagpur	Khunti	23° 07'	85° 27'	611	Open	Pop7	6	37-42
Kolhan	West Singhbhum	22° 36'	85° 43'	244	Dense	Pop8	6	43-48
Total = 04						**08**	**48**	

Materials and methods

Sample Collection, DNA isolation and Standardization of PCR conditions

The germplasm resources were collected from the different regions of Jharkhand where they existed in wild form. Leaf samples of 48 genotypes were collected from Hazaribag, Chatra, Giridih, Dhanbad and Bokaro (North Chotanagpur region), Khunti (South Chotanagpur region), West Singbhum (Kolhan region) and Palamau. Table 1 provides the details of the germplasm collected and used in the present study. The leaves

Data analysis

The amplified bands (reproducible bands only) were scored as '1' for presence and '0' for absence, and a rectangular binary data matrix was generated. Dendrogram based on data matrix was generated using NTSYS-pc version 2.02 K (Rohlf, 1998). Sequential, agglomerative, hierarchical, nested (SAHN) cluster analysis was performed on the data matrix using the unweighted pair group method with the arithmetic averaging (UPGMA) algorithm. Matrix based on population genetic data was also analysed using the software Popgene version 1.31

(Yeh *et al*., 1999) and Arlequin 3.5.2 (Excoffier *et al*., 2005). The Shannon index (I), Nei's genetic diversity (*h*), observed numbers of allele (*na*), effective numbers of alleles (*ne*), Nei's genetic identity and distance, number of migrants (N_m) between populations based on Nei's genetic variation (Gst) [$Nm = 0.5(1 - Gst) / Gst$] and the number of polymorphic loci for each population was estimated using Popgene version 1.31. Analysis of molecular variance (AMOVA) was used to estimate the variation among population using Arlequin 3.5.2, providing Fst values which represent the degree of genetic differentiation or population subdivision. The genotypes, populations and the region from where these genotypes were collected were subdivided into small groups on a predetermined criterion, to test and quantify between and within group variation. In order to confirm the Fst values, AMOVA data were submitted to 1023 independent permutations and P values lower than 0.05 were considered significant.

Results

A total of 120 amplification products were scored by 18 primers with an average frequency of 6.66 bands/ primer. Out of these 120 loci, 102 amplicon were polymorphic and 18 were monomorphic. Number of bands per primer ranged from 5 each for primers ISSR 4, ISSR 6, and ISSR 10, to 10 in primer ISSR 7. A list of no. of loci, no. of polymorphic bands, percentage polymorphism values generated by different primers is given in table 2. Figure 1 shows the representative ISSR fingerprinting profile of 48 genotypes.

The number of polymorphic bands within a population ranged from 39 (32.50%) for Khunti (pop7) to 69 (57.50%) for Hazaribag (pop1). The order of polymorphism was found to be population of Khunti< Chatra< West Singbhum< Giridih< Palamau< Bokaro< Dhanbad< Hazaribag. The relative degree of diversity, as indicated by Shannon's diversity index (I), across population ranged from 0.1824 in Khunti to 0.3292 in Hazaribag. The observed numbers of alleles at each locus in a population varied from 1.3250 in Khunti to 1.5750 in Hazaribag, while effective numbers of alleles ranged from 1.2169 in Khunti to 1.3976 in Hazaribag.

The mean value for Shannon's index (I), Nei's genetic diversity (*h*), observed numbers of allele (*na*), effective numbers of alleles (*ne*) was 0.4641, 0.3093, 1.8917, and 1.5302

Table 2. Total number of bands (n), number of monomorphic bands (mb), number of polymorphic bands (pb), and percentage of polymorphic bands (ppb) calculated for ISSR markers in *D. melanoxylon*

Primer	Primer Sequence (5'-3')	n	mb	pb	ppb
ISSR 1	AGAGAGAGAGAGAGAGC	07	02	05	71.4
ISSR 2	AGAGAGAGAGAGAGAGYA	06	02	04	66.6
ISSR 3	ACACACACACACACACC	06	00	06	100.0
ISSR 4	GAGAGAGAGAGAGAGAYT	05	00	05	100.0
ISSR 5	AGAGAGAGAGAGAGAGYT	08	00	08	100.0
ISSR 6	GAGAGAGAGAGAGAGAC	05	01	04	80.0
ISSR 7	GAGAGAGAGAGAGAGAT	10	02	08	80.0
ISSR 8	GAGAGAGAGAGAGAGAA	08	01	07	87.5
ISSR 9	CTCTCTCTCTCTCTCTA	07	00	07	100.0
ISSR 10	CACACACACACACACAA	05	02	03	60.0
ISSR 11	TCTCTCTCTCTCTCTCA	07	03	04	57.1
ISSR 12	AGAGAGAGAGAGAGAGYC	08	00	08	100.0
ISSR 13	GAGAGAGAGAGAGAGAYA	06	00	06	100.0
ISSR 14	CTCTCTCTCTCTCTCTRG	06	00	06	100.0
ISSR 15	GTGTGTGTGTGTGTGTYG	06	02	04	66.6
ISSR 16	TCTCTCTCTCTCTCTCRA	08	01	07	87.5
ISSR 17	ACACACACACACACACYT	06	00	06	100.0
ISSR 18	ATGATGATGATGATGATG	06	02	04	66.6
Total		120	18	102	85.0

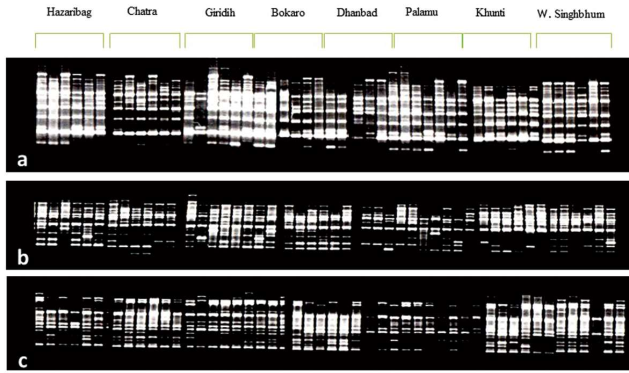

Fig. 1. Representative ISSR fingerprinting profile of 48 *D. melanoxylon* genotypes.

respectively (Table 3). The calculated gene flow was found to be 1.6533, indicative of a high amount of genetic exchange within the species. The overall genetic diversity value (H_t), 0.3093, intra-population variation (H_s) 0.1752, Co-efficient of gene differentiation (G_{st}) 0.4335 based on Nei's, were also estimated.

Apportionment of genetic variability by AMOVA revealed that most of the diversity (86.6%) was distributed between individuals within a population followed by among region (12.29%) and between populations within regions (1.11%), respectively (Table 4). Average f-statistics over all loci, Fst, Fct and Fsc were 0.13, 0.01 and 0.12, respectively.

Cluster analysis

The unweighted pair group mean arithmetic method (UPGMA) was employed to construct the dendrogram, based on the data matrix generated. The dendrogram thus generated, delineated the 48 genotypes into 5 major clusters (Fig. 2). Cluster 1 was represented by genotypes collected from Hazaribag. Cluster 2 was represented by the genotypes from Chatra, Cluster 3 was an admixture of genotypes from Khunti, Giridih, Dhanbad and Palamu. Cluster 4 was represented by genotypes collected from Giridih, Dhanbad and Bokaro. Two genotypes from Palamu and one each from Dhanbad and Bokaro were not part of the any group. Cluster 5 was represented by genotypes collected from West Singhbhum, and this connected as an out-group with the rest of the clusters. The neighbour-joining tree showed that terminal branches tended to be longer, suggesting that most of the ISSR distance in the data set exists between individuals than between groups.

Nei's genetic distances and genetic identity were also estimated for all the 8 populations. The genetic distance between populations

Table 3. Proportion of genetic diversity detected by ISSR markers for various populations of *D. melanoxylon*

Population	Sample size	*na*	*ne*	h	I	Number of polymorphic loci	Percentage polymorphism
Pop1	6	1.5750	1.3976	0.2247	0.3292	69	57.50
Pop2	6	1.3583	1.2438	0.2438	0.2033	43	35.83
Pop3	6	1.4500	1.2954	0.1706	0.2526	54	45.00
Pop4	6	1.5583	1.3498	0.1992	0.2965	67	55.83
Pop5	6	1.5583	1.3683	0.2032	0.3100	62	51.67
Pop6	6	1.4917	1.3453	0.1941	0.2837	59	49.17
Pop7	6	1.3250	1.2169	0.1237	0.1824	39	32.50
Pop8	6	1.3667	1.2676	0.1478	0.2147	44	36.67
Overall	48	1.8917	1.5302	0.3093	0.4641	102	85.00

na, observed number of alleles; *ne*, effective numbers of alleles; h, Nei's gene diversity; I, Shannon's diversity index.

Table 4. Apportionment of genetic diversity between and within populations of *D. melanoxylon* genotypes by AMOVA

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among regions	3	76.217	1.28	12.29
Among populations within regions	4	53.967	0.11	1.11
Within populations	40	353.817	9.07	86.6
Total	47	484.000	10.4671	

Significance tests (1023 permutations)

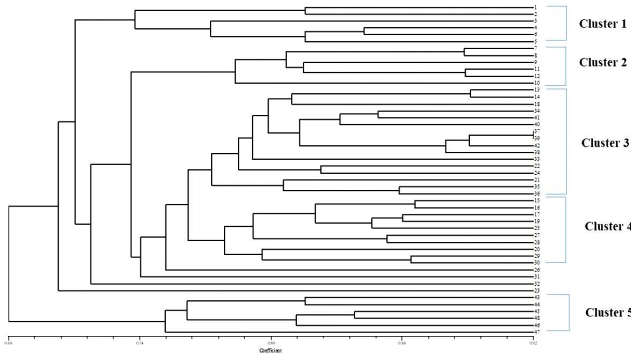

Fig. 2. UPGMA phenogram of 48 *D. melanoxylon* genotypes based on ISSR marker data.

Table 5. Genetic identity and genetic distance values detected by ISSR markers in *D. melanoxylon* populations.

pop ID	1	2	3	4	5	6	7	8
1	****	0.9503	0.8283	0.7966	0.7784	0.7685	0.7791	0.7945
2	0.0510	****	0.8399	0.8093	0.7919	0.7896	0.7694	0.7805
3	0.1884	0.1745	****	0.8819	0.7836	0.7899	0.7758	0.7428
4	0.2275	0.2116	0.1256	****	0.8309	0.8404	0.8092	0.7611
5	0.2505	0.2333	0.2439	0.1853	****	0.8700	0.8171	0.8194
6	0.2633	0.2363	0.2359	0.1739	0.1393	****	0.8859	0.8589
7	0.2496	0.2621	0.2538	0.2117	0.2020	0.1212	****	0.8673
8	0.2301	0.2479	0.2973	0.2731	0.1992	0.1521	0.1424	****

Nei's genetic identity above diagonal and genetic distance below diagonal

varied from 0.0510 between Hazaribag and Chatra's population to 0.2973 between West Singbhum and Giridih's population and genetic identity between population varied from 0.7428 between West Singbhum and Giridih's population to 0.9503 between Chatra and Hazaribag's population (Table 5) Genetic relationships between populations were further revealed by a UPGMA dendrogram (Fig. 3), using Popgene. Clustering analysis showed two clearly notable clusters for *Diospyros* populations. Cluster 1 was represented by populations Hazaribag, Chatra, Giridih and Dhanbad whereas, cluster 2 had Bokaro, Palamau, Khunti and West Singbhum' populations.

Discussion

With the estimated 80,000-100,000 different tree species across the globe, forests contribute immensely to the world's ecosystem as well as to the livelihood of rural people. For their livelihood, rural people depends mostly on non-timber produces (Porth and El-Kassaby, 2014). Kendu is one

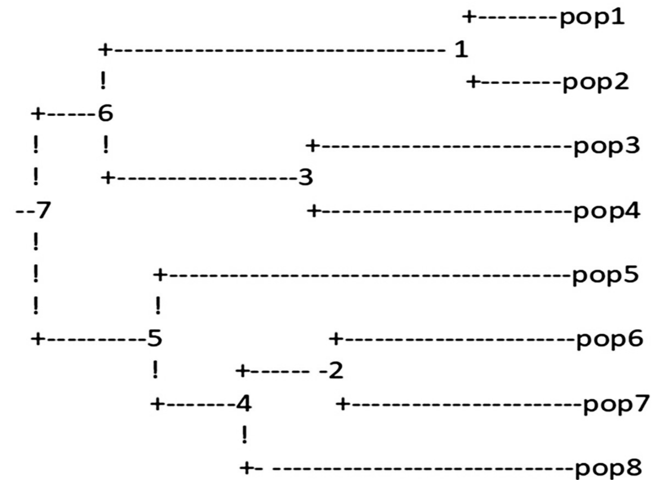

Fig. 3. Nei's genetic distance dendrogram of *D. melanoxylon* populations based on ISSR marker data.

such tree species which provides part time job (harvesting and trade of kendu leaves) to the people of Jharkhand, Chhattisgarh, Odisha, Madhya Pradesh, Tamil Nadu, etc.

It has been suggested that the life-history and reproductive pattern of a species (self or cross) are important determinants influencing the levels of genetic diversity, genetic divergence and genetic structure within and among plant populations (Loveless and Hamrick, 1984; Hamrick and Godt, 1996; Hamrick and Murawski, 1991). In addition the genetic diversity of natural populations can be influenced by a number of processes such as (a) mutation; (b) gene flow; (c) inbreeding; (d) natural selection; (e) the Wahlund effect; and (f) random genetic drift (Hartl and Clark, 2007). Genetic uniformity among plants of a species are also influenced by shared common ancestry and similar selective regimes (Schaal *et al.*, 1998).

A number of studies have demonstrated the high potential of ISSR markers for population and species level studies (Esselman *et al.*, 1999; Clausing *et al.*, 2000; Joshi *et al.*, 2000, Hui-yu *et al.*, 2005; Feng *et al.*, 2006). Theoretically, ISSR markers have a high capacity to reveal polymorphism (Zietkicwicz *et al.*, 1994), as these amplifies genomic regions between microsatellite areas that are potentially highly polymorphic, resulting into high polymorphism.

In the present study, the ISSR markers detected 85.0% polymorphism among 48 representative *D.*

melanoxylon germplasm of Jharkhand. The percentage of polymorphic bands in each population ranged from 32.50% in Khunti population to 57.50% in Hazaribag population. In terms of percentage polymorphism, the overall polymorphism, as expected for outcrossing perennial species, was high but the percentage polymorphism within each population was low. This result is supported by the AMOVA analysis of ISSR data, which showed that most of the genetic variation (86.6%) is distributed between individuals within the population. Similar pattern of geneticvariation has been previously reported for many other outcrossing tree species like *Populus tremuloides* (Liu and Furnier, 1993), *Pinus sylvestris* (Hui-yu *et al.*, 2005), *Eucalyptus globulus* (Jones *et al.*, 2007), *Dendropanax arboreus* (Esquivel *et al.*, 2010), *Madhuca indica* (Nimbalkar *et al.*, 2018), *Abies alba* (Teodosiu *et al.*, 2019).

Nei's gene diversity within population ranged from 0.1237 to 0.2247, and the overall Nei's gene diversity estimated was found to be 0.3093. The overall estimate is comparable with the tree species having similar life-history and characteristics. In widespread tree species, *Populus tremuloides*, Nei's gene diversity was 0.30, and even higher, 0.35, in *P. grandidentata* with restricted distribution (Liu and Furnier, 1993). For the very long-lived and widespread *Quercus petraea*, gene diversity was 0.298 (Lynch and Milligan, 1994). Nei's gene diversity was 0.22 in yet another widespread tree species, *Pseudotsugamen ziesii* (Aagaard *et al.*, 1998) and 0.19 in another widespread tree, *Moringa olifera* (Rajalakshmi *et al.*, 2019). Shannon's population diversity index unveiled maximum genetic diversity in Hazaribag population and least in Khunti population. The indirect estimate of gene flow (N_m) based on Gst was 1.6533.

The dendrogram exhibited the grouping of three out of eight population i.e; Hazaribag, Chatra and West Singhbhum into population specific clusters, and an admixture of genotypes from other areas. This can partly be explained by the fact that (i) in the recent past (100-200 years ago) this species might have formed a continuous stretch in these areas and now have become fragmented, and that (ii) the anthropogenic activities may have resulted into large amount of gene flow between Giridih, Dhanbad, Bokaro, Palamau and Khunti and hence, admixture of genotypes from these populations to form common clusters. Here, it will be interesting to note that Bokaro and Dhanbad are the two most urbanized cities of Jharkhand, while West Singhbhum is the most mined district of Jharkhand followed by Dhanbad. West Singhbhum was endowed with the most dense forest cover area in Jharkhand. However, due to intensive mining it has lost almost 20% of its dense forest area (fig. 4) during the year 1997 and 2017 (Bera *et al.*, 2018).

Our result exhibited that the genotypes from West Singhbhum differed considerably from other populations and was clustered as a separate group. Within the Jharkhnad's

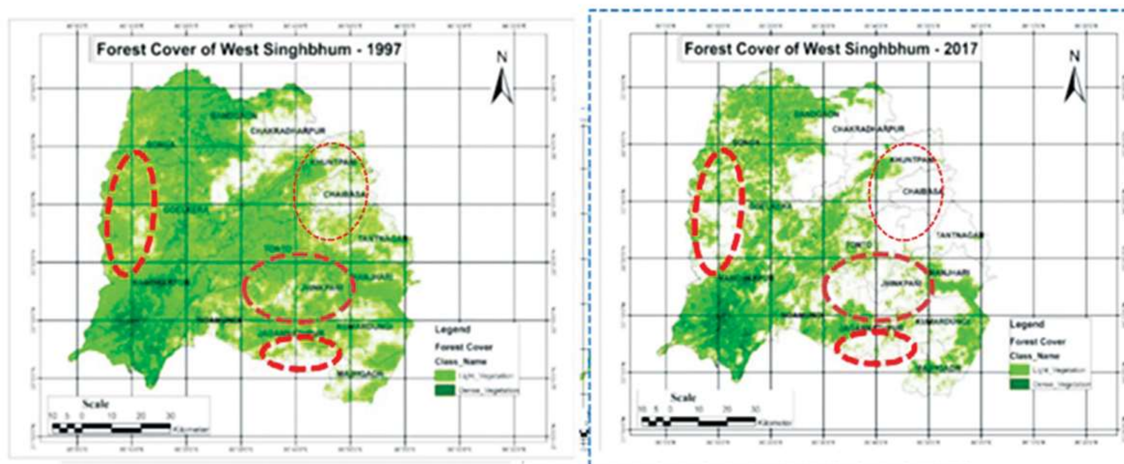

Fig. 4. Map showing the forest cover of West Singhbhum taken year from 1997-2017.
Note: Encircled region shows difference in the forest cover area from year 1997-2017
Source: Bera *et al.* 2018

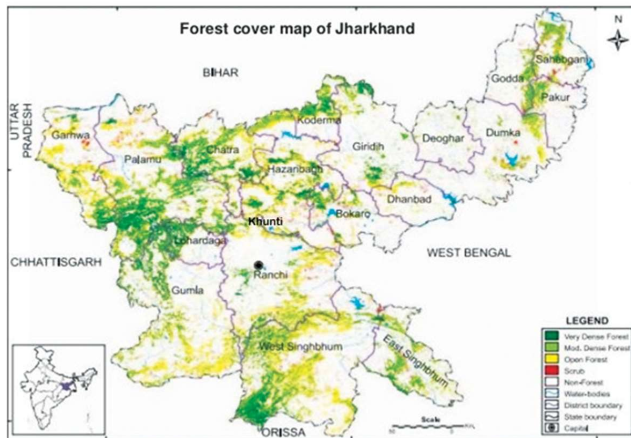

Fig. 5. Forest cover map of Jharkhand.
Source: ISFR 2017

geographical range (fig. 5), this region is clearly isolated from the central area of distribution and as such the distribution of this population can be classified as peripheral. The low level of similarity in the marginal population is consistent with the theory that marginal populations possesses lower genetic variation than in more central population (Levin, 1970). Further, it has been argued that populations which have remained isolated for long-time could accumulate private alleles reflecting their genetic differences because of isolation by distance (Prentice *et al.*, 2003). Due to its relatively low polymorphism content, trees from West Singhbhum which might be vulnerable to future environmental changes, requires special attention.

Conclusions

Fingerprinting of economically and medicinally important *D. melanoxylon* using ISSR markers, as presented here, has been reported for the first time. In spite of small sample size per population for a widespread outcrossing perennial species, ISSR markers detected considerable amount of genetic variability in *D. melanoxylon*. Based on the present study, three distinct hotspots of genetic diversity in *D. melanoxylon*, growing in Jharkhand: North Chotanagpur (Hazaribag, Chatra and Bokaro), Palamu and West Singhbhum, can be identified. Although, we acknowledge the limitation of interpretation of data with small sample size per population for a widespread outcrossing perennial species, this study can be seen as a stepping stone for future genetic studies of this economically and medicinally important species.

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