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Genetic diversity assessment in selected genetic resources of *Gmelina arborea* Roxb using RAPD markers

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Abstract
The genetic diversity and relationship among the fourteen accessions of *Gmelina arborea* were assessed using Random Amplified Polymorphic DNA (RAPD) markers. The RAPD profiles of all the accessions were compared and a total of 262 scorable bands were produced with fifteen primers, out of these 243 bands were polymorphic. The highest number of amplified products was observed in primers OPJ-19, OPM-02, OPN-02, OPN-05, OPN-16, OPN-18 and OPC-10 showed a lowest level of the polymorphic band. The similarity coefficient values were ranged from 0.58 to 0.74. The highest value similarity coefficient was found between the progenies of CPTs selected in Vazhachal and Kulathupuzha and the lowest values found between Vazhachal and Rosemala. Geographically closely related Gmelina accessions were also found to be related at the molecular level. A sizeable amount of inter-population diversity recorded, the Vazhachal and Rosemala accessions can be used as a parent in the Gmelina breeding program for improving productivity and wood quality.

Keywords: *Gmelina*, genetic diversity, RAPD, polymorphism and genetic similarity

Introduction
Gmelina arborea Roxb. is an fast-growing, multipurpose, indigenous tree species belonging to the family Lamiaceae. The species is naturally distributed between 5° to 30° N latitude and 70° to 110° E longitude (Chudnoff, 1979) [5] and grown in India, Pakistan, Nepal, Bangladesh, Sri Lanka, Myanmar, Thailand, Laos, Cambodia, Vietnam and southern provinces of China. However, in India the distribution of the species is extreamly scattered, mostly found within the mixed deciduous forests in association with Teak and also occasionally it is found in evergreen forests or semi-evergreen forest types (Troup, 1921; Lamb, 1970) [39, 20]. The wood is used for general construction, panelling, carriages, furniture, carpentry, boxes and plywood industries. It is also used to make musical instruments, artificial limbs, printing blocks and boat decking. The wood is also an excellent source for pulp and paper. The leaves are rich in protein with high fodder value. Roots and barks are used as main ingredients in many traditional medicines. The consumption of fruit gives a cooling effect to body and a decoction prepared from it is used as an ingredient in many traditional forms of medicines to cure anaemia, leprosy and ulcers (Tewari, 1995; Doat, 1976; Chothani *et al*., 2011) [38, 8, 4].
The nature and degree of divergence in the base population are pre-requisites for genetic improvement and development of conservation strategies (Ayad *et al*., 1995; Gradual *et al*., 1999) [1, 6]. Information from the field experiments on provenance evaluation is very valuable, because assessed adaptive genetic variation is still the best for breeding and conservation activities (Eriksson, 1995) [9]. The high levels of variability were found in Gmelina due to high out crossing nature of species, wide geographical distribution and adaptability to different agro climatic zones. The assessment of genetic diversity using morphological traits have been the oldest and used for certain management practices of germplasm and cultivars, where the cultivars have been identified on the basis of leaf, flower, fruit and other physical characteristics but environmental conditions has major impact on these traits. In Perennial trees identification of cultivar and assessing genetic diversity through morphological traits has several limitations (Purushotaman *et al.* 2008). Estimation of genetic diversity through morphological features is inefficient and inaccurate (Rahman *et al.* 2007) [28].

This can be overcome by direct identification of genotypes with DNA-based marker system. The use of biochemical and genetic markers for the identification of varieties offers a viable alternative method (Williams *et al.* 1990) [40-42]. DNA and protein-based markers are more authentic and unaffected by environmental factors (Dhanraj *et al.*2002) [7]. DNA-based genetic markers are integrated to play an important role in the future tree improvement programs of Gmelina. Molecular characterization of genotypes at the genetic level provides the basis for efficient maintenance, utilization and conservation of existing genetic diversity (Prakash *et al.* 2002) [26].

RAPD is a novel technique developed by Williams *et al.*, (1990) [40-42] for the analysis of genetic diversity, which is considered an efficient and cost-effective technique (Hadrys *et al.*, 1992). RAPD markers have been widely used in plant research for phylogenetic studies, genome mapping and genetic variation analysis both at intra- and inter-population levels (Li *et al.*, 2008). The technique has several advantages including simplicity, low cost, speed and lack of requirement for DNA sequence information (Williams *et al.*, 1990 [40-42]; Lopes *et al.*, 2007 [22]. This technique can also yield distinctive variable and multi fragment profiles (Isabel *et al.*, 1999) [15]. Assessment of genetic diversity using molecular markers is lacking in the Gmelina genetic resources of Tamil Nadu and Kerala. It is very much essential to study the genetic variability within and between populations of *G. arborea* through molecular markers and which are useful to identify the best genetic resources for tree improvement programme. Characterization of phenotypic and genetic diversity of Gmelina is essential since it facilitates the selection of the most suitable accessions for domestication and commercial cultivation. Understanding genetic structure of population is essential for conservation and sustainable management (Sun *et al.*1998) [36]. Successful application of RAPD and ISSR in the assessment of genetic diversity in tropical tree species has been well documented (Chalmers *et al*., 1992; Wilde *et al*., 1992; Gillies *et al.*, 1999; Esselmen *et al.*, 2000) [3, 41, 12, 10]. Assessment of genetic diversity would help for understanding the pattern and extent genetic diversity among the populations and would prove to be a potential gene source for gmelina crop improvement programmes. However, very limited studies have been undertaken to assess genetic variation through molecular markers in *G. arborea* in southern india. The aim of present investigations is to estimate the genetic diversity and assess the relatedness among fourteen accessions collected from different parts of Tamil Nadu and Kerala through RAPD markers.

Materials and methods

Survey and collection

The survey was conducted in the natural forest and plantation located in different parts of Tamil Nadu and Kerala. Fourteen Candidate Plus Trees (CPTs) of Gmelina were selected based on superiority in height, GBH, stem straightness, clear bole height, crown width, self-pruning ability and disease resistance. The point grading methods were adopted for the selection of Candidate Plus Trees. The seeds were collected from individual trees, labelled and transported to IFGTB, Nursery. Fruits were de pulped and washed in running water for extraction of seeds. The seeds were germinated in the raised raised nursery beds. About thirty days old seedlings were transplanted in a poly bags containing 2:1:1 (Red earth, sand and FYM) rooting medium. The young disease-free leaves of different accessions were used for the present study. Details of the accessions and geographical locations are presented in Table 1.

Table 1: Details on Geographical Locations of Selected Candidate Plus Trees of *G.arborea*

S. No	Tree Code	Place	Latitude	Longitude	Altitude (M)
1	TCBSG	Siruvani	N10˚56'39.5"	E76˚40'49.8"	433
2	TCAKG	Anaikkatti	N11˚03'23.5"	E 76˚46'53.7"	515
3	TESHG	Sathiyamangalam	N11˚36'13.2"	E 77˚04'16.8"	913
4	TESBG	Bargoor	N11°45'54.14"	E 77°33'14.10"	1056
5	TSYYG	Yercaud	N11˚46'26.1"	E 78˚11'23.5"	992
6	TDSSG	Sirumalai	N 10˚13'42.6"	E77˚58'43.9"	1088
7	TDKTG	Kodaikanal	N 10˚16'50.6"	E77˚38'41.4"	958
8	TTCKG	Courtallam	N8°56'19.77"	E 77˚11'59.9"	216
9	KEKBG	Bhoothathankettu	N10° 9'18.89"	E76°40'9.33"	60
10	KKTRG	Rosemala	N8°57'25.87"	E77° 9'12.23"	285
11	TPAKG	Pudukkottai	N10˚18'56.2"	E 79˚02'28.6"	75
12	KEMPG	Malayattoor	N10˚09'17.6"	E 76˚36'17.1"	131
13	KTAVG	Vazhachal	N10˚20'41.2"	E 76˚35'17.2"	350
14	KKKKG	Kulathupuzha	N08˚51'16.09"	E 77˚01'12.02"	175

Table 2: Estimation of quantity and quality of DNA isolated from progenies of *G. arborea*

S. No	Populations/Plantations	DNA quantity (ng/µL)	Optical density ratio ($A_{260/280}$)
1	Siruvani	28.4	1.64
2	Pudukkottai	29.0	1.71
3	Sathyamangalam	12.5	1.45
4	Bargur	9.4	1.22
5	Yercaud	7.8	1.39
6	Anaikatti	13.1	1.10
7	Sirumalai	15.0	1.25
8	Kodaikanal	7.0	1.49
9	Courtallam	7.7	1.48
10	Malayattoor	17.5	1.67
11	Bhoothathankettu	11.1	1.81
12	Vazhachal	20.8	1.42
13	Kulathupuzha	30.3	1.52
14	Rosemala	10.3	1.64
Mean		15.71	1.49

Isolation of genomic DNA

Genomic DNA was isolated using Qiagen DNeasy Plant mini kit (Qiagen, USA) following the manufacturer's protocol. Leaf samples weighing 100-120 mg were finely ground to powder using Liquid Nitrogen and transferred to sterile microfuge tube. The DNA was isolated with D Neasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany), following the protocol provided by QIAGEN with little modification.

The quality and yield of isolated DNA was verified with 0.8% agarose gel electrophoresis visualized by ethidium bromide staining. DNA was run alongside with k DNA-HindIII Digest (New England BioLabs Inc., Ipswich, MA, USA). The concentration of DNA samples were quantified using Nano Drop 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA). The concentration (ng/µL) and the optical density ratio ($A_{260/280}$) were recorded.

Selection of primer and RAPD – PCR program

The RAPD assays were performed using random 10-mer oligonucleotide primers from Eurofins Genomics India Pvt Ltd, Bangalore. PCR amplifications were carried out in 10 µL reaction volume using Veriti (Applied Biosystems, USA) thermal cycler. About 25 RAPD primers were tested and the 15 primers that produced clear and polymorphic amplification patterns were chosen for further analysis. The details of screened primer used for RAPD amplification are presented in Table 3. For each primer, the PCR amplification was carried out in 10 µl reaction volume mixture (2 µl Genomic DNA (50-60ng), 2.5 µlTaq Buffer (10X) with 1.5 mM MgCl2, 0.5 µl dNTP pre-mix (2.5 mM), 0.3 µl Taq polymerase (3 U/µl), 1.0 µl Primer (10 pmoles/ml) and 19.2µl Sterile Millipore water) as described by Williams *et al*. (1990) [40-42] in an Applied Biosystems, Verity 96 well, thermalcycler

PCR programme

The genomic DNA per population was pooled DNA from five randomly selected progenies per CPTs. The thermal cycler was programmed for an initial denaturation of 4 minutes at 94 °C, followed by 35 cycles of denaturation of 1 minute at 94 °C, 30 seconds of annealing at 37°C. The extension was carried out at 72°C for 2 minutes and final extension at 72 °C for 7 minutes and a hold temperature of 4 °C at the end.

The amplified DNA samples were mixed with 2.5µL of 6X DNA loading dye and then loaded into 1.5% Agarose gels with ethidium bromide (0.1%) along with 100bp DNA ladder (Thermo Scientific, USA). The gel was run at 80-100V for 2 hours in 1X TBE tank buffer, until all the amplified fragments are separated completely and then visualized on an ultraviolet trans-illuminator and documented through photographs.

The amplified products were scored as 1 for presence and 0 for absence at particular marker level generated by an accession and the marker. The binary data matrix table was computed for analysis using Numeric Taxonomy and Multivariate Analysis System (NTSYS-pc) version 2.02 software. The final score of each accession with the 15 primers were recorded. Genetic similarity for pair wise accessions were estimated based on similarity for qualitative data format (Jaccard, 1908). A similarity matrix was constructed and subjected to cluster analysis following the Unweighed Pair Group Method with Arithmetic average (UPGMA) method using NTSYS PC version 2.0 software.

Results and Discussion

Genomic DNA was isolated using Qiagen DNeasy Plant mini kit (Qiagen, USA) in the progenies of 14 CPTs. The quantity of DNA (ng/µL) and the optical density ratio ($A_{260/280}$) were recorded is detailed in Table 2. In the present investigation, the quantity of DNA from 15 accessions of *Gmelina arborea* is ranged from 7.0 ng/µL to 30.3 ng/µL. The highest quantity of DNA was obtained from progenies of KKKG-1 (30.3 ng/µL), TPAAG-2 (29.0 ng/µL) and lowest DNA yield of 7.0 ng/µL was recorded from TDKTG-1 progeny.

Randomly Amplified Polymorphic DNA (RAPD) techniques have been effectively utilized to identify polymorphism in several forest tree species (Mohapatra and Singhal, 2000) [23]. It is widely used because of the high frequency of polymorphism, rapidity, technical simplicity and requirement of low quantity of DNA (Subudhi and Huang, 1999) [35]. RAPD has been successfully used to study the genetic diversity in trees *viz*., teak (Keiding *et al*. 1986) [18], eucalyptus (Keil & Griffin 1994) [19], oil palm (Shah *et al*. 1994) [33], mango (Ravishankar *et al*. 2000) [31] and plum (Shimida *et al*. 1999). Hence the RAPD markers proved to be very useful for estimating the genetic diversity of *Gmelina arborea*.

Random Amplified Polymorphic DNA (RAPD) analysis was carried out and all the progenies produced a large number of distinct fragments with each primer. Initially twenty five primers (Operon Technologies, USA) were tested and out of which fifteen primers yielded clear polymorphic and reproducible bands (Table 2). The amplified products varied in number and intensity among the fourteen progenies. The size of the amplified fragments ranged from 130 bp to 3270 base pairs, (bps). The total number of products generated and the levels of polymorphism observed by the 15 RAPD primers are given in Table 3. RAPD analysis of 14 progenies representing different regions of Tamil Nadu and Kerala generated 262 distinct bands. Among 262 bands amplified by 15 primers, 243 (92.7%) were polymorphic and 19 (7.30 %) were monomorphic. The average number of bands per primer was 17.46 and average number of polymorphic bands per primer was 16.2.

The number and percentage of polymorphic RAPD bands obtained are detailed in Table 5. The percent polymorphic bands varied from 78.95 percent to 100 percent with the average polymorphic percentage of 92.45 %. Primers OPJ-19, OPM-02, OPN-02, OPN-05, OPN-16 and OPN-18 were found to be highly polymorphic and showed 100 percent polymorphism. On the other hand, primer OPC-10 showed lower polymorphic band of 78.95%. The polymorphic information content (PIC) was ranged from 0.25 (OPC-10) to 0.43 (OPN-10) with an average found to be 0.34. The PIC was highest in case of OPN-05 (0.43), OPN-16 and OPN-18 (0.41) whereas the primers OPC-10 (0.25), OPC- 03 and OPE-20 (0.29) showed the lowest PIC. Certain primers such as OPE -19 (0.34) and OPE-4 (0.33) showed the medium level of PIC. Gmelina is a highly cross pollinated tree species and heterozygous in nature. The polymorphism levels observed in present investigation was fairly high and it indicating that a wide and diverse genetic base existed between progenies of different CPTs selected in Tamil Nadu and Kerala. This variation due to out crossing nature and diverse origin of selected CPTs.

The high level of polymorphism generated by all RAPD markers in this study dramatically higher compared to the results of previous studies conducted in Thailand (Kanawapee *et al*., 2011) [17], Bangladesh (Hasan and Raihan, 2015) [14], Iraq (Abdi;-razzal Tahir, 2014) [37], and India (Rajani *et al*., 2013) [30]. Similar study was conducted in *Tectona grandis* by Parthiban (2001) [25] using 17 arbitrary primers and observed 74 % of polymorphism. It was also reported in *Azardirachta indica* (Ranade and Farooqui, 2002) [29], *Acacia auriculiformis* and *A. mangium* (Nguyen *et al.*, 2004) [24]. The genetic similarity matrix was constructed using the RAPD bands to assess the genetic relatedness among the 14 accessions of *G arborea* (Table 4).The Jaccard's similarity matrix values

ranged from 0.45 to 0.74. The highest similarity value of 0.74 was found between the accessions of Vazhachal with Kulathupuzha followed by Anaikatti with Yercaud and Sirumalai with Bargur (0.69). However the lowest similarity co-efficient was observed between the progenies Vazhachal with Rosemala (0.45). The Jaccard's similarity matrix was used to generate dendrogram for quantifying the level of polymorphism between the accessions. The dendrogram was constructed based on simple matching co-efficients, taking into account of the presence or absence of DNA bands. The phonetic tree was constructed by selecting the Unweighted Pair Group Method with Arithmetic Average (UPGMA) and it is presented in Fig. 3. The UPGMA clustering algorithm based on RAPD data grouped a fourteen accessions into three clusters at co-efficient level of 0.60. Similar findings have been reported by Parthiban (2001) [25] in teak with estimated levels of genetic variation between 0.4 and 0.1. The present investigation also supports the observation made by Chalmers *et al*., (1992) [3] in *Gliricidia* with a estimated levels of genetic variation between 0.4 and 0.7. Tarachand *et al*., (2015) reported the similarity co-efficient ranged between 0.07 and 0.64 among 12 accessions of *Garcinia cambogia*. Umesh Kanna (2001) also observed the same trend in *Madhuca latifolia*. Raza *et al*. (2018) [32] reported that, the 86.34% polymorphism and 13.65% monomorphism was observed using twenty RAPD markers to estimate the genetic diversity among ten sunflower genotypes.

All the accessions originated from the CPTs selected in Tamil Nadu were grouped into cluster-I. The cluster I was further sub divided into two groups. Siruvani, Anaikkatti, Sathyamangalam, Kodaikanal, Courtallam, Bargur, Sirumalai and Yercaud accessions were grouped in a sub cluster I A and Pudukkottai accession was grouped in I B. Similarly Most of the accessions collected from Kerala were grouped into cluster II. The cluster II was further sub divided in to two sub-clusters. Malayattoor, Vazhachal and Kulathupuzha accessions were grouped in a sub cluster II A and Bhoothathankettu accessions was grouped in sub cluster II B. The Cluster III had a Rosemala accessions selected from Kerala. The *G. arborea* trees were distributed throughout in Tamil Nadu and Kerala. Major clusters were reflecting the same clustering pattern of superior trees of same geographic origin (Fig. 17). It is contrary with the findings of Ganesh Ram (2000) [11] and Balaji (2000) [2] who estimated genetic diversity in *Azadirachta indica* and *Eucalyptus tereticornis* using RAPD markers and stated that distribution of the genotypes did not group based on geographical origin.

Table 3: Level of polymorphism of various primers used for RAPD analysis

S. No.	Primer	Band size range	Total number of bands	Number of polymorphic bands	Percentage of polymorphism	Polymorphic information content (PIC)
1	OPE-20	130-1890	12	10	83.33	0.31
2	OPC-10	141-2767	19	15	78.95	0.25
3	OPE-04	249-2130	17	15	88.24	0.33
4	OPE-20	259-2102	16	14	87.50	0.29
5	OPJ-19	181-1413	11	11	100.00	0.30
6	OPM-02	148-3270	17	17	100.00	0.35
7	OPN-02	204-2427	14	14	100.00	0.31
8	OPN-03	275-2769	15	13	86.67	0.29
9	OPN-05	305-1413	13	13	100.00	0.43
10	OPN-16	211-2650	19	19	100.00	0.41
11	OPN-18	169-2181	23	23	100.00	0.41
12	OPP-18	172-1885	24	22	91.67	0.36
13	OPP-19	136-2721	24	23	95.83	0.31
14	OPX-01	217-1952	18	15	83.33	0.37
15	OPD-05	162-1719	20	19	95.00	0.31
Mean			17.47	16.2	92.745	0.34
Total			262	243		

Table 4: Genetic Similarity coefficient matrix among populations/plantations based on RAPD marker

	Siruvani	Pudukkottai	Sathyamangalam	Bargur	Yercaud	Anaikatti	Sirumalai	Kodaikanal	Courtallam	Malayattoor	Bhoothathankettu	Vazhachal	Kulathupuzha	Rosemala
Siruvani	1.000													
Pudukkottai	0.607	1.000												
Sathyamangalam	0.660	0.611	1.000											
Bargur	0.611	0.660	0.676	1.000										
Yercaud	0.634	0.592	0.592	0.641	1.000									
Anaikatti	0.706	0.611	0.679	0.691	0.698	1.000								
Sirumalai	0.649	0.576	0.645	0.695	0.618	0.630	1.000							
Kodaikanal	0.660	0.580	0.626	0.637	0.615	0.695	0.637	1.000						
Courtallam	0.695	0.576	0.637	0.634	0.611	0.615	0.656	0.714	1.000					
Malayattoor	0.569	0.534	0.534	0.546	0.508	0.550	0.561	0.504	0.538	1.000				
Bhoothathankett	0.504	0.515	0.515	0.511	0.489	0.553	0.489	0.538	0.534	0.592	1.000			
Vazhachal	0.553	0.511	0.550	0.531	0.531	0.534	0.538	0.534	0.592	0.603	0.660	1.000		
Kulathupuzha	0.584	0.504	0.565	0.492	0.523	0.542	0.546	0.511	0.576	0.679	0.607	0.740	1.000	
Rosemala	0.546	0.511	0.504	0.523	0.477	0.496	0.523	0.489	0.492	0.466	0.469	0.458	0.466	1.000

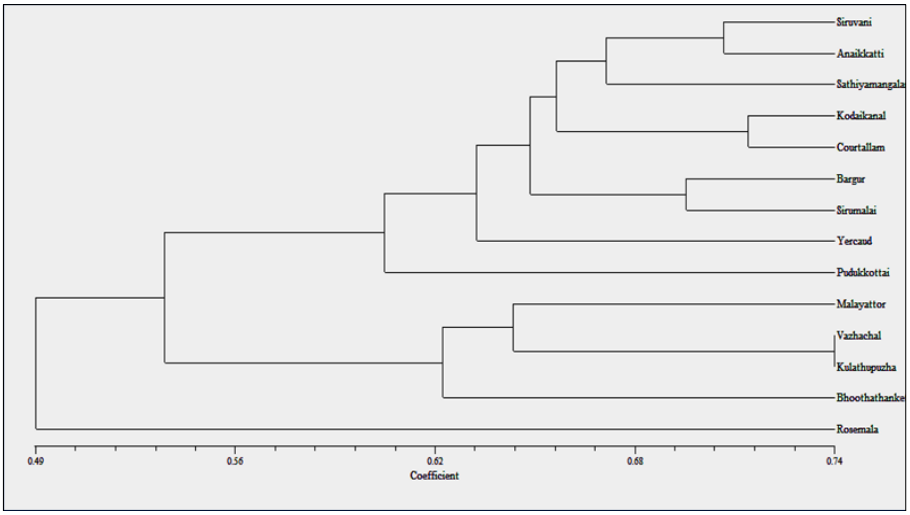


Fig 1: UPGMA dendrogram of G. arborea as revealed by RAPD markers

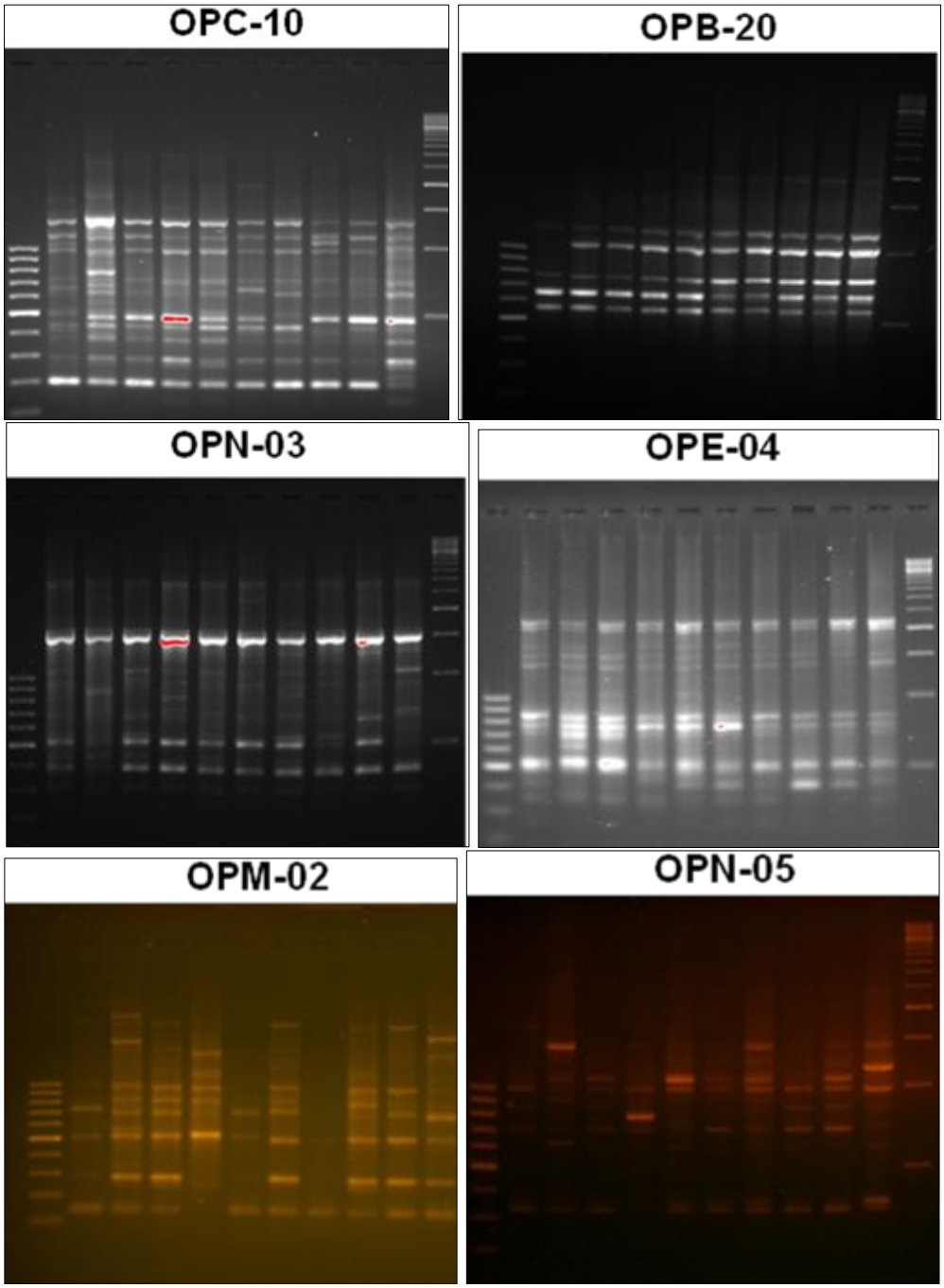


Fig 17: RAPD profiling of OPB-20, OPC-10, OPN-03, OPE-04, OPM-02, OPN-05 primers in *G. arborea*

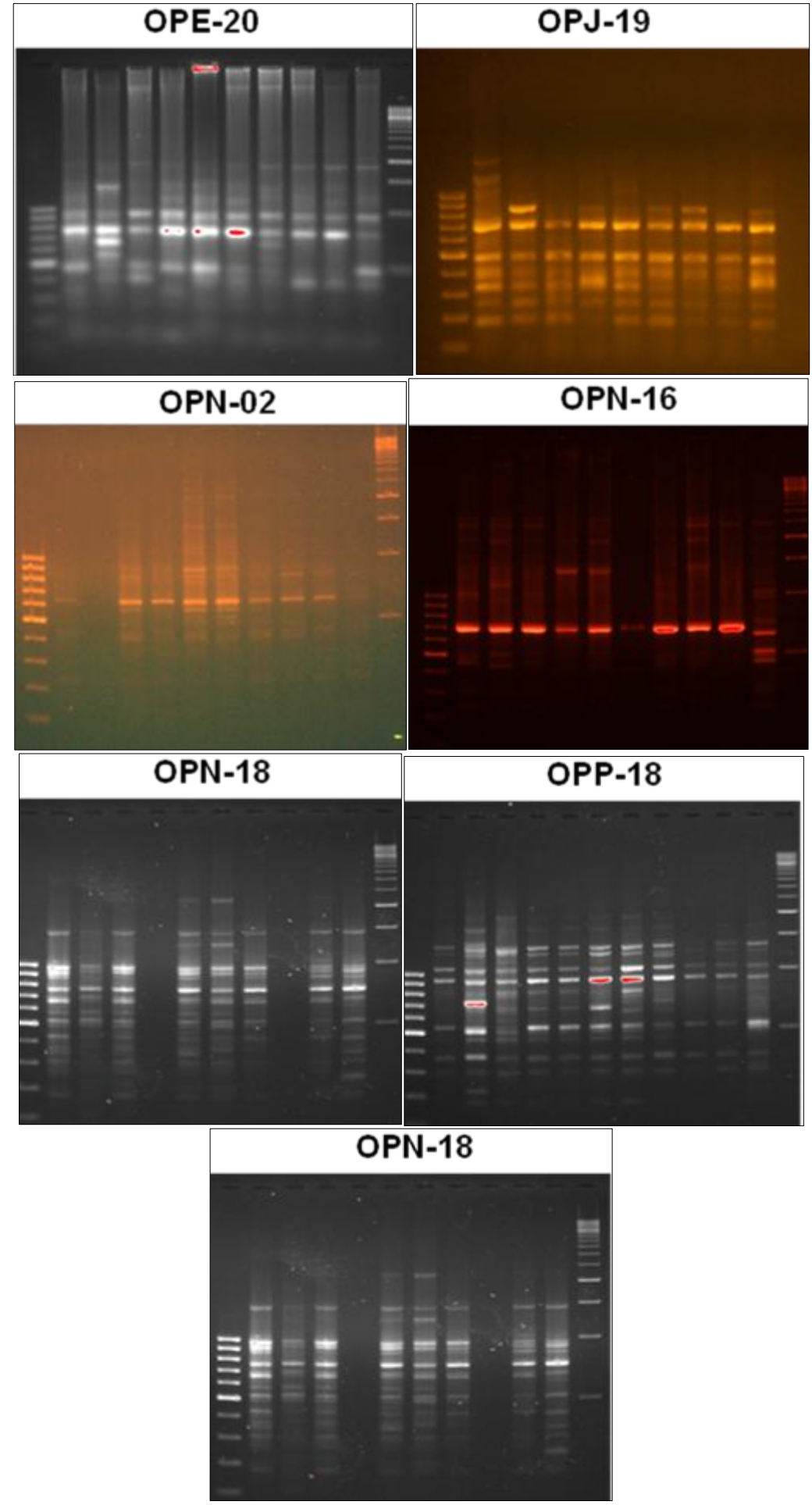
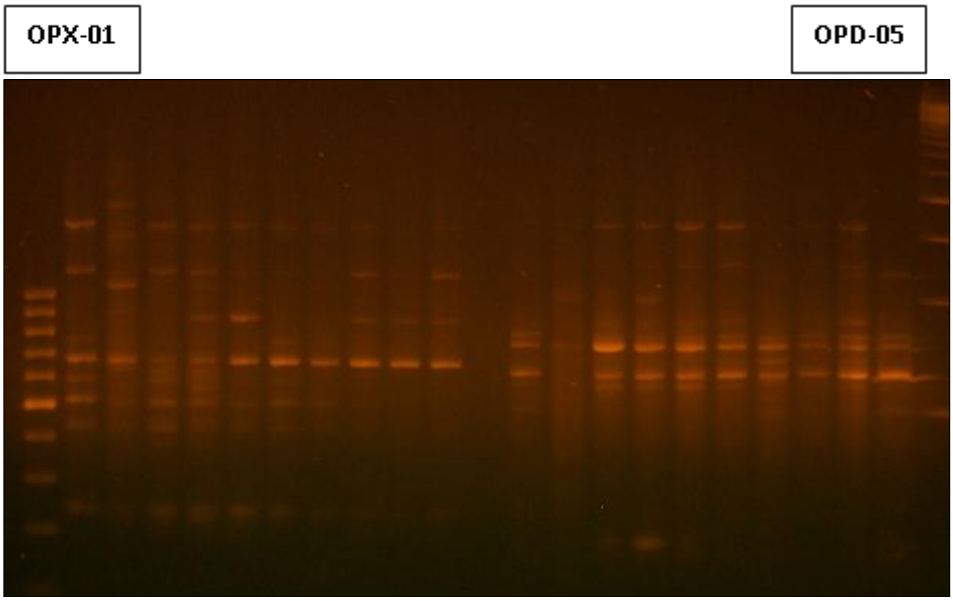


Fig 1: RAPD profiling of OPE-20, OPJ-19, OPN-02, OPN-16, OPN-18 and OPP-18 primers in *G. arborea*



Lane 1 – 100bp ladder, Lane 2-11 – DNA samples of *G.arborea*, Lane 12 – 500bp ladder

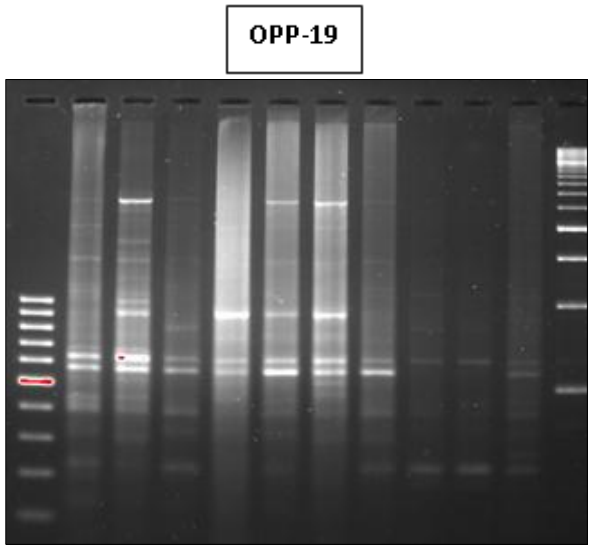


Fig 19: RAPD profiling of OPX-1, OPD-5 and OPP-19 primers in *G. arborea*

Conclusion

The Random Amplified Polymorphic DNA (RAPD) marker analysis has revealed presence of high level of genetic variation among the fourteen accession of Gmelina. However differences among the geographical locations were significant and accession collected in Tamil Nadu and Kerala were grouped separately. The study demonstrated that the RAPD markers can be effectively utilized for assessing the genetic relationship among *Gmelina arborea* genetic resources.

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