Full Length Research Paper

Application of rep-PCR as a molecular tool for the genetic diversity assessment of *Jatropha curcas*

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Jatropha curcas L., a non-edible Euphorbiaceae oil-rich crop cultivated in subtropical/tropical countries, has gained global attention as a promising renewable resource for biodiesel production. Rep-polymerase chain reaction (PCR) was used to investigate the genetic diversity of 15 populations of *J. curcas* L. Distinct populations of the plant growing wildly in Mauritius were characterised using three molecular markers random-amplified polymorphic DNA (RAPD), repetitive extragenic palindromic (REP), and BOX. Furthermore, to confirm that the amplicons obtained with rep-PCR were derived from mitochondrial genomes, six randomly chosen bands were cloned and sequenced to demonstrate that the amplified products were mitochondrial genome-specific. The average polymorphism information content (PIC) values were 0.329 and the average percentage of polymorphic loci obtained were 89.28 for BOX primer, followed by RAPD (83.41), and REP (55.81) among the different populations with the percentage polymorphic loci ranging from 13.95 to 100. The homology recorded clearly indicated that the amplified products were mitochondrial genome-specific. Rep-PCR provides a quick and cheap method to study diversity at the mitochondrial level in plants.

Key words: Genetic diversity, *Jatropha curcas,* rep-polymerase chain reaction (PCR).

INTRODUCTION

Jatropha curcas L., an economical non-edible energy crop of the family Euphorbiaceae, has received great attention in recent years for its utilization in biodiesel production, rehabilitation of wasteland, and rural development (Kumar and Sharma, 2008; Koh and Ghazi, 2011; Pandey et al., 2012). However, despite enormous and up-to-date research made to develop *J. curcas* as an energy profitable crop, the absence of improved cultivars and lack of agronomic knowledge refrain the full exploitation of the plant's potential. Knowledge in genetic divergence between *J. curcas* populations around the world is vital for the selection of parent plants aiming at the breeding and selection of progenies with superior traits of interest as well as for the maintenance of genetic diversity in improvement programmes and germplasm banks (Sun et al., 2008).

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Several studies have conceded that molecular markers are fundamental technological instruments that help in improving selection and gaining more insight about the divergence of the phenotypic level among the different populations. Amplified fragment length polymorphism (AFLP), random-amplified polymorphic DNA (RAPD), and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) are molecular markers that have been used in several studies to assess genome-wide variability of *J. curcas* (Basha and Sujatha, 2009, Pamidimarri et al., 2009; Sunil et al., 2011). Comparative studies have shown a very high genetic uniformity even among accessions from different continents; however, the only genetic variability in *J. curcas* was observed in Mexicans accessions (Basha et al., 2009; Ambrosi et al., 2010; Maghuly et al., 2014). Basha and Sujatha (2009) evaluated 42 *J. curcas* accessions from different regions in India. These authors used random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers to determine the genetic diversity and reported on the immediate need to improve the genetic base of the Indian *J. curcas*.

Organellar genomes, chloroplast DNA (cpDNA), and mitochondrial DNA (mtDNA) have remarkably been accepted in recent years as markers to assess maternal/or paternal gene flow mainly explained by their uniparental mode of inheritance (Grivet and Petit, 2003). Repetitive sequence based polymerase chain reaction (rep-PCR) technique has been devised for the characterisation of bacteria and also widely employed to distinguish species, strains, and serotypes among others. The technique devised by Lupiski and Weinstock (1992) used three specific primers, designated BOX, enterobacterial repetitive intergenic consensus (ERIC), and repetitive extragenic palindromic (REP) designed to match the conserved sequences distributed in diverse bacterial genomes. REP sequences also known as elements were first described in *Escherichia coli* and *Salmonella typhimurium* operons. These sequences have the ability to form stable stem-loop structures which has a regulatory role in transcriptional termination, mRNA stability, and chromosomal organization in bacteria (Versalovic et al., 1991). Other related families of repetitive elements, such as ERIC and BOX sequences, have been exploited in the molecular identification of bacteria pathogenic to plants. Three types of PCR (known as Rep-PCR) based on these elements have been favoured mainly because it was quick and more costly-effective than with other methods, such as AFLP and RFLP. These primers amplify genomic regions located between repetitive sequences and have proven extremely useful in the study of microbial diversity. Rep-PCR application in plant was first reported by Dwivedi et al. (2005) where this technique was used for the determination of the different cytoplasmic male sterility (CMS) lines of *Brassica juncea* and for identifying mitochondrial genome diversity in safflower (*Carthamus tinctorius* L.) as well as their wild relatives.

This study was undertaken to evaluate the genetic diversity of *J. curcas* from the different regions in the subtropical Island of Mauritius. Rep-PCR as an innovative and potential tool for studying plant's diversity was used to access the *Jatropha* intra-populational variance using RAPD, BOX and REP molecular markers.

MATERIALS AND METHODS

The samples were collected from 15 areas based on their geographical locations. Fresh leaves of wildly distributed plants were collected at 10 diffrenet places in the same location. Initially, 150 samples of fresh leaves of the wildly distributed *J. curcas* were used for the research work (Figure 1).

Isolation and quantification of genomic DNA

Fresh, young and tender leaves were selected and frozen at -80°C. Genomic DNA was extracted from the leaves crushed to a fine powder by cetyltrimethylammonium bromide (CTAB) method. The leaf tissues were ground with a mortar and pestle to a fine powder using liquid nitrogen. Five grams of the leaves powder were homogenized in 20 ml of extraction buffer (2% w/v) CTAB, 20 mM ethylenediaminetetraacetic acid (EDTA), 2% (v/v) polyvinyl pyrrolidone (PVP), 1.4 M NaCl, 100 mM Tris–HCl (pH 8.0) and 1% (v/v) β-mercaptoethanol and were incubated at 65°C for 45 min. The supernatant was treated with RNase A (10 mg/μl), incubated at 37°C for 30 min and twice extracted with chloroform:isoamylalcohol (24:1 v/v). The DNA was precipitated with isopropanol and washed twice with 70% (v/v) ethanol. The pelleted DNA was air dried and resuspended in 500 μl of sterile millipore water and stored overnight at -20°C. The purity of the extracted DNA was determined by taking the ratio of the absorbance at 260 and 280 nm.

PCR Protocols

RAPD

All the PCR reactions were carried out in 25 μl volumes containing 50 ng of template DNA, 200 mM 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, and 10 pmol of primer of RAPD primers. 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, 2 min elongation at 72°C and a final extension at 72°C for 7 min. After completion of the amplification, 2.5 ml 10X blue dye was added to the samples, and the amplified DNA was analysed on 2% agarose gel in 5X TBE buffer at 70 V for 4 h. Out of 40 RAPD primers selected, only the following 11 primers were successfully amplified: Opb 3 catccccctg, Opc 16 cacactccag, Opd 3 gtcgccgtca, Ope 2 ggtgcgggaa, Opf7 ggagtactgg, Oph 9 tgtagctggg. Oph 12 acgcgcatgt, Opi 20 aaagtgcggg, Opj 5 ctccatgggg, Opo 9 tcccacgcaa, and Opp 3 ctgatacgcc

Rep-PCR

Amplification was carried out in a 20 μl reaction mixture consisting of 50 ng/μl genomic DNA, 10X PCR reaction buffer containing 15 mM MgCl$_2$, 10 pM primer, 2.5 mM of each dNTP and 3 U/μl of Taq DNA polymerase (Genei, Bangalore, India). Amplification was performed in a thermal cycler (Eppendorf, Germany) PCR machine.
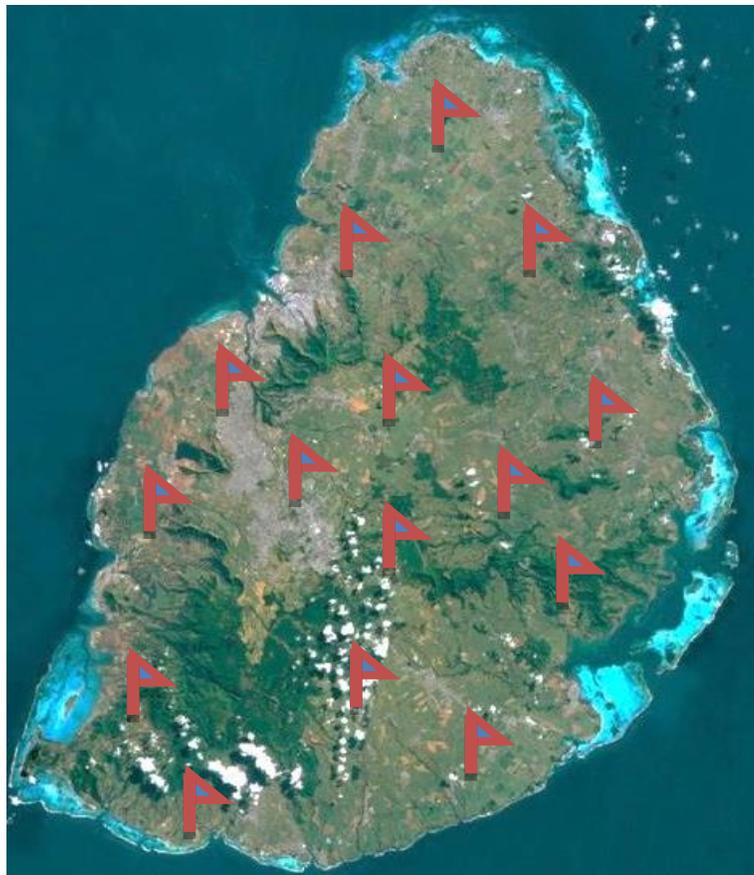
North Samples codes	
Pamplemousses	N11-N110
Triolet	N21-N210
Point aux Piments	N31-N310
South	
Souillac	S11-S110
Chemin Grenier	S21-S210
Mahebourg	S31-S310
Central	
Curepipe	C11-C110
Rose Belle	C21-C210
Floreal	C31-C310
East	
Centre de Flacq	E11-E110
Camp de Masque Pave	E21-E210
Saint Julien D'Hotman	E31-E310
West	
Flic en Flac	W11-W110
Tamarin	W21-W210
Bamboos	W31-W310

Figure 1. Samples collection location and codes around Mauritius Island.

The PCR conditions were 94°C for 3 min, followed by 45 cycles of DNA amplification 20 s at 92°C, 1 min at 52°C for BOXA1R primer and 1 min at 38°C for REP primers and 8 min at 68°C and 15 min incubation at 68°C, respectively.

(1) BoxAIR: CTACGGCAAGGCGACGCTGACG,
(2) REP1R:IIIICGICGICATCIGGC/REP1I: ICGICTTATCIGGCCTAC

All PCR reactions were carried out in triplicate. The PCR products were run on 2% (w/v) agarose gel for 7 to 8 h at constant voltage (2 V/cm). To ensure reproducibility and representativeness of the experiment, 10 PCR runs were carried out.

Cloning

To confirm that the amplicons obtained from rep-PCR were of mitochondrial genomes, six representative amplicons obtained using the two (BOX and REP) primers were extracted using a gel extraction kit (Qiagen, Hilden, Germany) and cloned into the PCR cloning vector, pMiniT Vector (NEB PCR Cloning Kit, New England Biolads, UK). The amplicons cloned ranged from 575 bp to 1 kb in length. All the clones were sequenced at Inquaba (Pretoria, South Africa) using the Forward Primer: 5´ ACCTGCCAACCAAAGCGAGAAC 3´ and Reverse Primer: 5´ TCAGGGTTATTGTCTCATGAGCG 3´ available in the vector. The sequences obtained were subjected to basic local alignment search tool (BLAST) analysis to determine their identity.

Data scoring and statistics analysis

For scoring and analysis of data from the three molecular markers (RAPD, BOX and REP markers), bands which were clear, unambiguous and reproducible were scored and data scoring was carried out using a binary number system for '1' as presence and '0' as absence of fragment (band) for primers. The allele frequency of the populations and the basic statistics were generated for the different generated scored profiles using the POPGEN32 software and the difference between the populations were determined using the analysis of molecular variance (AMOVA- GenAIEx version 6.5).

Phylograms were constructed using the maximum parsimony method. Furthermore, to test the robustness of the phylo, the indices were bootstrapped 10000 times using PAUP version 4.0b (Swofford, 2002). The information content of each markers was computed as $PiC_i = 2\ f_i(1-f_i)$; where, f_i is the frequency of the amplified allele (band present) and $1- f_i$ is the frequency of the null allele.

RESULTS

The dendrogram generated based on the maximum parsimony cluster analysis for the three markers separated the populations in different number of clusters: RAPD (14), BOX (6), and REP (11). 46% of the populations formed the largest cluster for REP primer

followed by BOX primer (36.7%). For REP marker, the cluster with low genetic diversity grouped most of individuals collected in three distinct regions (North 70%, South 66.7%, and West 53.33%) and the remaining clusters with the higher genetic diversity were composed with basically populations collected in Central and East, but it also had samples collected in north, south and west, where it is possible to select individuals to be included in breeding programs. Most of the clusters for the BOX and RAPD showed high genetic variation (Figure 2).

Genetic variation among the three primers used revealed that the BOX showed the lowest variation (0.051), whereas REP primer had the highest genetic variation (0.429) (p < 0.001) which represents high differentiation among population (Table 1) as compared to RAPD primer (0.079). REP primer analysis of molecular variance (AMOVA) showed that 42.89% of the total variation corresponds to those between populations, intra-populational, and the remaining to variation among populations. The dendrogram generated based on the unweighted pair group method with arithmetic (UPGMA) cluster analysis for the three markers separated the populations in different number of clusters: RAPD (4), BOX (6), and REP (9). 71.33% of the populations formed the largest cluster for BOX primer followed by RAPD primer (60.67%) (Figure 2).

A total of 140 well-defined and visible bands for the three primers were scored on agarose gel for the molecular analysis. The minimum and maximum numbers of polymorphic bands observed for each primer were 41 and 56, respectively. The highest polymorphism information content (PIC) value (0.3712) was obtained for BOX primer (Table 2). For the computation and description of the genetic variation among the fifteen sampled regions from allele frequencies, Hardy Weinberg equilibrium was assumed. For the fifteen different regions, the number of alleles averaged to 1.5 and 100% total polymorphic loci. All bands were polymorphic, the percentage polymorphic loci ranged from 13.95 to 100 for the primers (Table 3). The lowest percentage of polymorphic loci was for REP primer, whereas BOX had the highest polymorphic percentage. Considering all five populations, a 100% polymorphic loci were recorded for BOX primer. Populations from S3 presented the lowest Shannon diversity index overall (REP: 0.06, BOX: 0.277, RAPD: 0.368), respectively. The highest number of locus per allele was observed for the primer BOX and the percentage of polymorphic loci was the highest. Population from E2 and E3 presented the highest percentage of polymorphic loci (100%) using BOX primer, followed 55.81 and 83.7% with REP primer (Table 3).

To further confirm the amplified regions are of mitochondrial origin, four sequences were randomly cloned and sequences. The blast results showed that the sequences, coded for proteins that were located in the mitochondrial region. The homology of the second REP

primer sequence result was in the chloroplast region, which again maybe due to horizontal gene transfer as mitochondrial genomes known to acquire sequences from chloroplast (Figure 3).
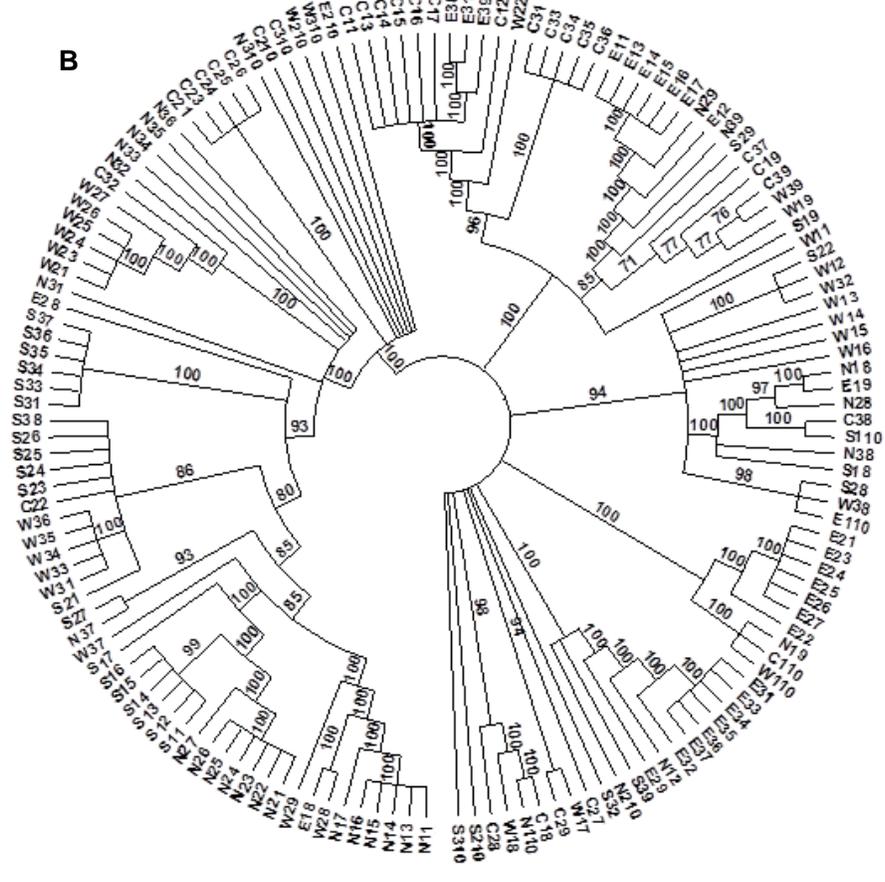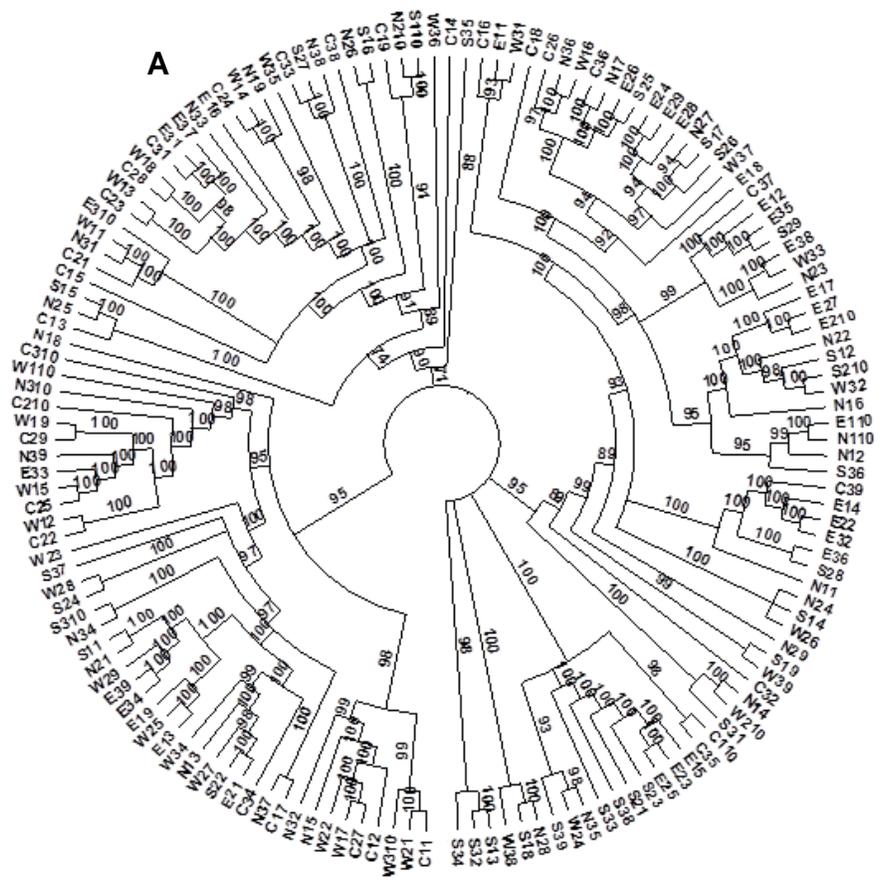
DISCUSSION

Genetic variation among *J. curcas* population is a pre-requisite for commercialising the plant. Intensive selection programme among the plant population represents a crucial part for the development of new viable economical cultivars. Molecular markers play a fundamental role in the study of genetic variability in plants. There are several DNA markers such as RAPD, SSR, ISSR, and AFLP that have been used for the fingerprinting of plant germplasm (Shen et al., 2010). Diversity in the *J. curcas* has been investigated previously based onagromorphological traits, biochemical traits, molecular markers including isozymes. Genetic variability of the plant still needs to be established as different marker systems previously used showed high degree of homozygosity (Heller, 1996).

This study demonstrates that REP and BOX primers are convenient and inexpensive solutions for screening wild plant species as compared to RFLP or RAPD techniques. RFLP requires high technical and resource demands (Fukunaga and Kato, 2003), whereas RAPD requires a large number of random primers to be screened to identify the polymorphic ones. Therefore, PCR-based method that targets the varied interspersed repeat sequences found in mitochondrial genomes has an immense importance in assessing chondriome diversity.

The markers generally applied in prokaryotes were successfully applied in eukaryotes and have equally proved to be very effective. The degree of polymorphism was comparable to primers generally employed for diversity studies. The PIC values ranged from 0.3067 to 0.3712 and were higher than in SSR and EST-SSR markers (average 0.216 ± 0.078 standard deviation) with respect to those in SNP markers (average 0.272 ± 0.108 standard deviation) recorded. The results are also in accordance with the findings of Tatikonda et al. (2009) and Grativol et al. (2011) showing PIC values ranging from 0.20 to 0.34 in *J. curcas*; although for BOX primer, the PIC was higher than what the authors obtained.

Furthermore, the level of polymorphism found in our work (100%) was superior to those already reported previously. Pamidimarri et al. (2009) used RAPD and AFLP for the analysis of species of *Jatropha* and the mean percentage of polymorphism was 68.48 and 71.33%, respectively, and determined 69.57% polymorphisms in Indian selected germplasm. *J. curcas* populations of Chiapas-Mexico (Medina et al., 2013; Yi et al., 2010) produced 52 useful markers with 81.18% polymorphism, 88% in elite germplasm collection of *J.*

A

B

Figure 2. Relationships among the populations of *J. curcas* L. in the 15 different regions based on the maximum parsimony method for **A)** BOX, **B)** REP and **C)** RAPD markers (Bootstrap=10000 using PAUP Version 4.0b).

Table 1. AMOVA between and within population for RAPD, REP, and BOX Primers.

Source	df	SS	MS	Variance components	% of total variation	p	PHIst
BOX							
Between population	14	211.933	15.138	0.530	5.107	<0.001	0.051
Within population	135	1328.600	9.841				
REP							
Between population	14	425.533	30.395	2.682	42.89	<0.001	0.429
Within population	135	482.200	3.572				
RAPD							
Between population	14	151.413	10.815	0.500	7.929	>0.001	0.079
Within Population	135	784.500	5.811				

df: Degree of freedom; SS: sum of squares; MS: sum of mean squares; p: level of significant for the estimate of genetic variation based on 1000 permutations. PHIst statistic: Genetic variation (Wright statistics). The analyses were done using GenAIEx 6.5.

Table 2. Markers selected for *J. curcas* molecular analysis.

Marker	Total number of bands	Polymorphic bands	Polymorphism (%)	PIC value
Box	56	56	100	0.3712
RAPD	41	41	100	0.3083
REP	43	43	100	0.3067

PIC: Polymorphism information content.

Table 3. Descriptive statistics of three markers for the 15 different regions in Mauritius.

Population	na*			h*			I*			P*		
	REP	BOX	RAPD	REP	BOX	RAPD	REP	BOX	RAPD	REP	BOX	RAPD
Cl	1.6152 (0.4822)	1.7500 (0.4369)	1.7561 (0.4348)	0.1856 (0.1574)	0.2079 (0.1475)	0.2254 (0.1664)	0.2953 (0.2372)	0.3327 (0.2187)	0.3525 (0.2369)	65.12	75	75.61
C2	1.3488 (0.4822)	1.9464 (0.2272)	1.8780 (0.3313)	0.0809 (0.1282)	0.3571 (0.1361)	0.2649 (0.1381)	0.1348 (0.2004)	0.5288 (0.1759)	0.4151 (0.1929)	34.88	94.64	87.80
C3	1.5349 (0.5047)	1.8393 (0.3706)	1.8730 (0.3313)	0.1330 (0.1459)	0.2911 (0.1641)	0.2268 (0.1260)	0.2182 (0.2242)	0.4404 (0.2251)	0.3692 (0.1772)	53.49	83.93	87.80
E1	1.7674 (0.4275)	1.9643 (0.1873)	1.8730 (0.3313)	0.2247 (0.1561)	0.3568 (0.1133)	0.2571 (0.1459)	0.3541 (0.2264)	0.5328 (0.1459)	0.4043 (0.1990)	76.74	96.43	87.80
E2	1.5581 (0.5025)	2.0000 (0.00)	1.7805 (0.4191)	0.1437 (0.1515)	0.4493 (0.0632)	0.2537 (0.1738)	0.2335 (0.2304)	0.6398 (0.0696)	0.3885 (0.2435)	55.81	100	78.05
E3	1.8372 (0.3735)	2.0000 (0.00)	1.8537 (0.3578)	0.2349 (0.1418)	0.4411 (0.0707)	0.2551 (0.1423)	0.3742 (0.2202)	0.6309 (0.0773)	0.4008 (0.2013)	83.72	100	85.37
N1	1.7442 (0.4415)	1.8571 (0.3531)	1.8049 (0.4012)	0.2009 (0.1528)	0.2807 (0.1469)	0.2746 (0.1696)	0.3224 (0.2225)	0.4316 (0.2071)	0.4166 (0.2386)	74.42	85.71	80.49
N2	1.7674 (0.4275)	1.8571 (0.3531)	1.9512 (0.2181)	0.2205 (0.1458)	0.2296 (0.1355)	0.2839 (0.1264)	0.3505 (0.2171)	0.3698 (0.1913)	0.4449 (0.1638)	76.74	85.71	95.12
N3	1.4651 (0.5047)	1.9107 (0.2877)	1.8049 (0.4012)	0.1191 (0.1459)	0.3279 (0.1496)	0.2468 (0.1579)	0.1941 (0.2259)	0.4908 (0.1991)	0.3845 (0.2243)	46.51	91.07	80.49
S1	1.5581 (0.5025)	1.9107 (0.2877)	1.7073 (0.4606)	0.1488 (0.1604)	0.2600 (0.1274)	0.1907 (0.1587)	0.2390 (0.2391)	0.4127 (0.1747)	0.3056 (0.2305)	55.81	91.07	70.73
S2	1.3953 (0.4947)	2.0000 (0.00)	1.9024 (0.3004)	0.1014 (0.1446)	0.4204 (0.0918)	0.2976 (0.1431)	0.1647 (0.2200)	0.6073 (0.1036)	0.4556 (0.1939)	39.53	100	90.24
S3	1.1395 (0.3506)	1.6607 (0.4778)	1.8293 (0.3809)	0.0377 (0.1064)	0.1707 (0.1468)	0.2312 (0.1440)	0.0600 (0.1614)	0.2778 (0.2224)	0.3688 (0.2048)	13.95	66.07	82.93
W1	1.4651 (0.5047)	1.9821 (0.1336)	1.6585 (0.4801)	0.1140 (0.1431)	0.3504 (0.1283)	0.1927 (0.1675)	0.1874 (0.2205)	0.5248 (0.1553)	0.3033 (0.2461)	46.51	98.21	65.85
W2	1.6977 (0.4647)	1.9464 (0.2272)	1.9268 (0.2637)	0.1879 (0.1532)	0.3555 (0.1333)	0.3376 (0.1487)	0.3022 (0.2271)	0.5268 (0.1733)	0.5034 (0.1937)	69.77	94.64	92.68
W3	1.4419 (0.5025)	1.7679 (0.4260)	1.9024 (0.3004)	0.1098 (0.1437)	0.2471 (0.1666)	0.2888 (0.1457)	0.1798 (0.2213)	0.3806 (0.2387)	0.4447 (0.1956)	44.19	76.79	90.24
									Average	55.81	89.28	83.41

Mean sample size=10; Number of observed alleles per locus (na); Nei's gene diversity (h); Shannon's Information (I) and percentage of polymorphic loci (P). The Hardy-Weinberg equilibrium was assumed and the parameters were compiled using Popgen32. Figures in brackets are ± standard deviation.

curcas from India, and 26.99% using seeds from cultivated populations in China (Ganesh et al., 2008).

In this study, rep-PCR was used to delve intraspecific diversity within *J. curcas* populations around Mauritius. The basis was to use a multi loci primer which targets the whole genome, such as RAPD markers, then compare the diversity obtained to only mitochondrial repeat markers BOX and REP. Diversity at mitochondrial level obtained from only BOX and REP proved to be very informative as compared to RAPD. Although BOX and REP target diversity only in the mitochondria, the genetic variation among the three primers used revealed that BOX showed the lowest variation (0.074), whereas REP primer had

the highest genetic variation (0.429) (p < 0.001) which represents high differentiation among population (Table 1) as compared to RAPD primer(0.051). Hence, the ten percent of mitochondrial repeats are as informative and useful in diversity study as a genome based primer (Dyall et al., 2004).

Conclusions

Rep-PCR could be used effectively to study the variability in genetic diversity. Sequencing of randomly chosen PCR products demonstrated clearly that the PCR products obtained were derived from plastid genomes. Thus, the rep-PCR

technique seems to combine the advantages of economy and adequate resolution for distinguishing different chondriome. Furthermore, it has added advantages over RFLP technique, as it does not require high amount of good quality DNA. The same set of primers could also be used to analyse diversity in chloroplast genomes, as plastids are also derived from ancient prokaryotic endosymbionts and their genomes show similarity to prokaryotes. This diversity information can represent a great resource for crosses and breeding programmes.

Conflict of Interests

The authors have not declared any conflict of
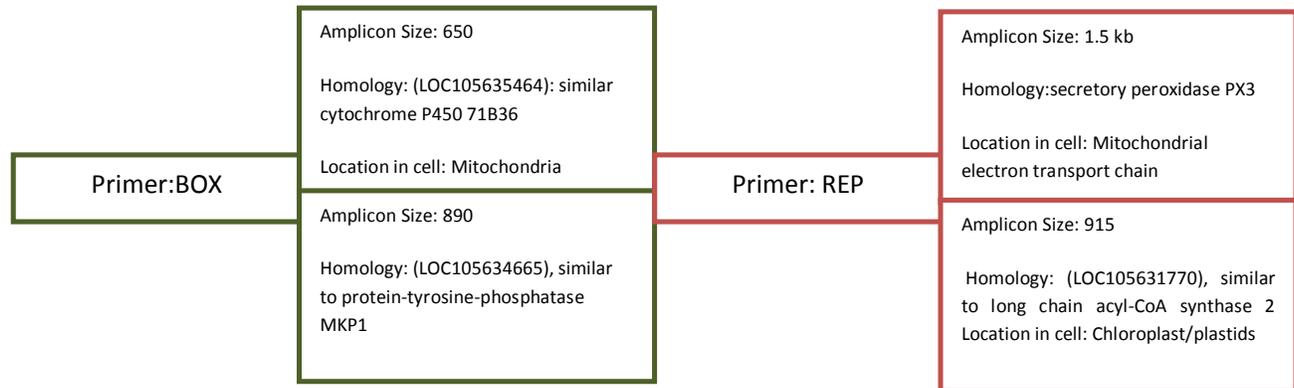
Figure 3. Blast results of clone sequences from both BOX and REP primers.

interests.

Abbreviations

PCR, Polymerase chain reaction; **RAPD,** random-amplified polymorphic DNA; **REP**, repetitive extragenic palindromic; **PIC,** polymorphism information content.

REFERENCES

Ambrosi DG, Galla G, Purelli M, Barbi T, Fabbri A, Lucretti S, Sharbel TF, Barcaccia G (2010). DNA markers and FCSS analyses shed light on the genetic diversity and reproductive strategy of *Jatropha curcas* L. Diversity 2(5):810-836.

Basha SD, Francis G, Makkar HPS, Becker K, Sujatha M (2009). A comparative study of biochemical traits and molecular markers for assessment of genetic relationships between *Jatropha curcas* L. germplasm from different countries. Plant Sci. 176:812-823.

Basha SD, Sujatha M(2009). Genetic analysis of *Jatropha* species and interspecific hybrids of *Jatropha curcas* using nuclear and organelle specific markers Euphytica 68(2):197-214.

Dwivedi A, Kumar VD, Prakash S, Bhat SR(2005). rep- PCR helps to distinguish different alloplasmic cytoplasmic male sterile lines of *Brassica juncea* Plant Sci. 168:1083-1087.

Dyall SD, Brown MT, Johnson PJ (2004). Ancient invasions: from endosymbionts to organelles. Science 304:253-257.

Fukunaga K, Kato K (2003). Mitochondrial DNA variation in foxtail millet *Setariaitalica* (L.) P. Beauv. Euphytica 129:7-13.

Ganesh RS, Parthiban KT, Senthil KR, Thiruvengadam V, Paramathma M (2008). Genetic diversity among *Jatropha* species as reveled by RAPD markers. Genet. Resour. Crop Evol. 55(6):803-809.

Grativol C, Lira-Medeiros CF, Hemerly AS, Ferreira PCG (2011). High efficiency and reliability of inter-simple sequence repeats (ISSR) markers for evaluation of genetic diversity in Brazilian cultivated *Jatropha curcas* L. accessions. Mol. Biol. Rep. 38:4245-4256.

Grivet D, Petit RJ (2003) Chloroplast DNA phylogeography of the hornbeam in Europe: evidence for a bottleneck at the outset of postglacial colonization. Conserv. Genet. 4:47-56.

Heller J (1996). Physic nut-*Jatropha curcas* L, in: Promoting the Conservation and Use of Underutilized and Neglected Crops, International Plant Genetic Resources Institute, Rome, Italy.

Koh MY, Ghazi TIM (2011). A review of biodiesel production from *Jatropha curcas* L. oil. Renew. Sustain. Energy Rev.115:2240-2251.

Kumar S, Sharma S (2008). An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. Ind. Crop Prod. 28:1-10.

Lupiski JR, Weinstock GM (1992). Short, interspersed repetitive DNA sequences in prokaryotic genomes. J. Bacteriol.174:4525-4529.

Maghuly F, Jankowicz-Cieslak J, Pabinger S, Till BJ, Laimer M (2014) Geographic origin is not supported by the genetic variability found in a large living collection of *Jatropha curcas* with accessions from three continents. Biotechnol. J. 10(4):536-551.

Medina IO, Sanchez-Gutierrez A, Adrano-Anaya L, Espinosa-Gracia F, Nú~nez-Farfan J, Salvador-Figueiroa M (2013). Genetic diversity in *Jatropha curcas* populations in State of Chiapas, Mexico. Diversity 4:641-659.

Pamidimarri DVNS, Mastan SG, Rahman H, Reddy MP (2009) Molecular characterization and genetic diversity analysis of *Jatropha curcas* L. in India using RAPD and AFLP analysis. Mol. Biol. Rep. 37:2249-2257.

Pandey VC, Singh K, Singh JS, Kumar A, Singh B, Singh R (2012). *Jatropha curcas*: A potential biofuel plant for sustainable environmental development. Renew. Sustain. Energy Rev.16:2870-2883.

Shen J, Jia X, Ni H, Sun P, Niu S, Chen X (2010). AFLP analysis of genetic diversity of *Jatropha curcas* grown in Hainan, China. Trees 24:455-462.

Sun Q, Li L, Li Y, Wu G, Ge X (2008). SSR and AFLP Markers Reveal Low Genetic Diversity in the Biofuel Plant *Jatropha curcas* in China. Crop Sci. 48:1865-1871.

Sunil N, Sujatha M, Kumar V, Vanaja M, Basha SD, Varaprasad KS (2011). Correlating the phenotypic and molecular diversity in *Jatropha curcas* L. Biomass Bioenergy 35:1085-1096.

Tatikonda L, Wani SP, Kannan S, Beerelli N, Sreedevi TK, Hoisington DA (2009). AFLP-based molecular characterization of an elite germplasm collection of *Jatropha curcas* L., a biofuel plant. Plant Sci. 176(4):505-513.

Yi C, Zhang S, Liu X, Bui HTN, Hong Y (2010). Does epigenetic polymorphism contribute to phenotypic variances in *Jatropha curcas* L. BMC Plant Biol. 10:259.