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Abstract

Due to the increasing popularity of *Jatropha curcas* as a feedstock for biodiesel, generating, non-toxic and high yielding varieties of the plant requires genotypic characterization towards identifying breeding lines. There is little information on the phylogenetic relationships between its global accessions and species. Assessing genetic variation by RAPD, AFLP and combinatorial tubulin based polymorphism (cTBP) in 38 *J. curcas* accessions from 13 countries on 3 continents revealed narrow genetic diversity while the 6 *Jatropha* species from India exhibited pronounced genetic diversity indicating higher possibilities of improving *J. curcas* by interspecific breeding. The relatively unexplored cTBP approach was an efficient molecular tool. Presence of the co-carcinogenic phorbol esters (PE) in the seeds, seed-cake and biodiesel is undesirable. We report initial success in obtaining interspecific F_1 and back cross (BC_1) plants with low PE and improved agronomic traits. Further BC populations will lead to varieties with targeted traits. Despite the limited genetic diversity within *J. curcas* accessions, appreciable variability exists in important phenotypic, physiological and biochemical traits such as seed size, water use efficiency and seed oil content respectively. This implicates fundamental epigenetic regulatory mechanisms and posits *J. curcas* as a unique system to study them.

Key words: *Jatropha curcas*, biodiesel, phorbol esters, genotyping, breeding, phenotypic plasticity, epigenetics.

Introduction

Jatropha curcas is becoming an increasingly popular plant (Fearless, 2007; Nature editorial 2007) for its proposed value in the biodiesel, biopharmaceuticals, cosmetics and biopesticides industry (Gubitz *et al*., 1999; Kumar & Sharma, 2008). Meagre genotypic characterization, sub-optimal agronomic practices, limited information on the genome of *J. curcas* and a scarce number of its isolated genes require major research initiatives in agronomy, breeding and molecular biotechnology of *J. curcas* for it to live up to its potential (Popluechai et al., 2008). Gressel (2008) recommended the transgenic approach for improvement of biofuel crops including *J. curcas*. *Agrobacterium*-mediated transformation of *J. curcas* was recently reported (Li *et al*., 2008), allowing transgenic *J. curcas* to be generated for the desired traits. The 'omics' based technologies can allow fast-track genome sequencing and relevant gene identification.

The major constraint in the widespread acceptance of *Jatropha* is the presence in seed/oil, of toxins and antinutrient factors (ANFs) such as curcins, saponins, protease inhibitors, phytate and the co-carcinogenic phorbol esters (PE; Makkar *et al*., 1997). The seeds are inedible and harmful, leading to *J. curcas* seeds also being called black vomit nut and purge nut and its oil being called hell oil or oil-infernale. *J. curcas* cannot be an economically viable feedstock without reduction/elimination of the ANFs and varietal improvement for oil yield (Gressel, 2008; Kohli *et al*., 2009). Elimination of toxins such as curcins was recommended as one of the primary targets (Gressel, 2008) because curcin being similar to ricin has an immediate toxic, often fatal effect (Gressel, 2008). Lin *et al*., (2003) achieved the cloning, expression and characterisation of the *Jatropha* curcin as a ribosome inactivating protein, and Luo *et al*., (2006) described its useful antitumor effects. Makkar & Becker, (1999) proposed a detoxification method for curcin but whether such processing is cost effective or not would depend on the scale of the operation and the prevalent market forces. Importantly

however, exposure to *J. curcas* PEs is of equal, if not higher concern due to their co-carcinogenic effect that becomes evident much later after exposure. Currently up to 55% of the original PE content is retained in the biodiesel after the degumming and deodorization processes and even after additional deacidification and bleaching (Haas & Mittelbach, 2000). Seed PEs may additionally limit the commercial value of *J. curcas* by making the otherwise protein-rich seed-cake unsuitable as an animal feed. Recently Devappa & Swamylingappa (2008) reported detoxification of the seed-cake. Another method reducing seed-cake PE to undetectable levels (sensitivity of the method: 5 ppm) is being considered for patent protection. The performance of carp fish fed the detoxified seed meal at a level of 75% replacement of fishmeal protein is at par with soyabean meal (HPSM - personal communication). However, human exposure to PEs up to the stage of generating the seed-cake still remains in both methods. The reduction of PEs through the RNAi approach would require the yet unidentified *J. curcas*-specific PE synthesis genes because the diterpene biosynthesis pathway involved in PE production comprises divergent genes. Additionally, the pathway is similar to that of plant hormones gibberellins and abscisic acid, thus demanding precision in manipulation. Incidentally, certain *J. curcas* accessions from specific regions in Mexico are edible and assessed to contain no or a minimal amount of PE that is not harmful to humans (Makkar *et al*., 1998). The use of such accessions, including the ones with variable yield parameters, in conventional breeding could be a beneficial parallel approach.

To gain maximum benefit from breeding efforts, identification of breeding lines with target traits in genetically polymorphic background is desirable. As discussed later, existing information on genetic diversity of *J. curcas* is confined to accessions from India and reveals a narrow genetic base. No data exists for comparison of accessions from different parts of the world. We used RAPD, AFLP and/or cTBP to assess genetic polymorphism between 38 *J. curcas* accessions from 13 countries on 3 continents. Six additional *Jatropha* species from India were also assessed. Results indicated high

degree of monomorphism in *J. curcas* accessions obtained from around the world. Only the accessions from Mexico and Costa Rica exhibited polymorphism but were still nearly 70% similar to other accessions in our analysis, indicating limited use of intra-specific breeding programs even if global accessions were used. Additional *Jatropha* species exhibited polymorphism indicating *J. gossypifolia* and *J. integerrima* as closer to *J. curcas*. Interspecific breeding of *J. curcas* x *J. integerrima* was successfully achieved earlier (Sujatha & Prabakaran, 2003). *J. integerrima* exhibits certain agronomically undesirable traits such as relatively thin stems and the seeds contain relatively low oil and high levels of PE. However, Sujatha & Prabakaran (2003) reported hybrid plants with intermediate and acceptable phenotype in terms of stem size and seed shattering. In the present study we report additional interspecific crosses and further analysis of back cross (BC) of the hybrids to *J. curcas* that identified plants with improved yield traits and reduced PE. However, the challenge remains to obtain and select advanced BC generation plants, not necessarily introgressed for, but introduced with heterozygosity for the desirable traits. This may not be difficult to achieve because hybrid plants that flower within 5-6 months instead of 16-24 months were earlier identified and maintained (Sujatha & Prabakaran, 2003).

Our results demonstrate the utility of cTBP as a simple and efficient genotyping tool. More importantly, the cTBP results with parental and hybrid plants indicate that increased heterozygosity in F_1 plants of interspecific hybrids may indeed explain the wide range, and thus improved plants, for the desirable traits. We obtained marked phenetic variability in morphological, biochemical and physiological traits such as seed size, total oil content and composition and water use efficiency respectively in *J. curcas* accessions with high genetic similarity. The phenomenon of phenetic differences in genetically identical organisms is well known (Lewontin & Goss, 2004). Epigenetic mechanisms for such differences in an undomesticated plant have been explored in the popular model *Arabidopsis* (Blodner *et al*., 2007). However, *J. curcas* as a novel and undomesticated plant

system uniquely exhibits naturally widespread genetic monomorphism at a global level. An understanding and the importance of the epigenetic/stochastic events within such a unique genome (allelome) have not been explored. With the construction of gene libraries and its genome sequencing underway (ISAAA, 2008), *J. curcas* may be a useful system to further define the molecular mechanisms underlying pronounced phenotypic variability.

Materials and Methods

Plant and seed material.

The number of accessions of *J. curcas* and additional *Jatropha* species obtained from around the world are listed in Table S1. Plants were either grown in the fields in Hyderabad, India (Latitude 17n20 and longitude 78e30, average temperature 27 ^{0}C, average relative humidity 57% and soil type red sandy loam) and Chiang Rai, Thailand (Latitude 19n54 and longitude 99e50, average temperature 26 ^{0}C, average relative humidity 76% and soil type sandy/clayey loam) or in controlled environment chambers. In the latter case plants were grown at 28ºC on a 16 h photoperiod of light intensity (PAR) of 300 µE $m^{-2}s^{-1}$ and a relative humidity of 40%. The *Jatropha* interspecific hybrids and backcrosses were generated as described earlier (Sujatha & Prabakaran, 2003)

DNA extraction RT-PCR

Jatropha DNA was extracted as described earlier (Basha & Sujatha, 2007). For RT-PCR the Invitrogen RT-PCR kit was used as per the manufacturer's instructions using 1 µg of total RNA with oligo dT and Superscript II reverse transcriptase for first strand cDNA synthesis. RT-PCR products were quantified using Fluoro spectrophotometer (BioRad)

RAPD, AFLP and cTBP analysis.

Each RAPD reaction mixture of 20 µl contained 50 ng DNA template, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton-X100, 0.2 µM 10-mer primer (Operon Technologies), 100 µM of each of 4 dNTPs, 2 mM $MgCl_2$, and 0.75 unit *Taq* DNA polymerase (Bioline). Amplifications were performed in a Thermal Cycler (Perkin-Elmer) programmed for an initial incubation at 94°C for 3 min, followed by 35 cycles of 30 sec at 94°C, 45 sec at 36°C and 1 min 30 sec at 72°C. The samples were incubated at 72°C for 4 min and held at 4°C prior to analysis. Amplified products were resolved on 0.8 % (w/v) agarose gel electrophoresis. Gels were stained with 0.5 µg/ml ethidium bromide solution and visualized on a UV transilluminator (Bio-Rad). GenRuler^{TM} DNA Ladder (Fermentas) was used as a standard DNA marker.

The AFLP procedure was carried out as per the manufacturer's instructions (Invitrogen). Thirty-two primers were used for amplification. PCR products were analyzed as described earlier (Lin *et al*., 1996). The silver staining process included fixing the gel in 10% (v/v) acetic acid for 20 minutes, rinsing in de-ionized water (3x5 minutes), staining for 30 minutes in a solution containing 0.2% (w/v) silver nitrate and 0.015% (v/v) formaldehyde. The stained plate was rinsed with de-ionized water for 10 seconds and developed in a cold (4–10°C) developer solution containing 3% (w/v) sodium carbonate (BDH Analar grade), 0.015% (w/v) formaldehyde, and 0.002% (w/v) sodium thiosulphate until the DNA bands became visible. The gel was rinsed with distilled water and air-dried. RAPD or AFLP bands were recorded and cluster analysis performed using the Neighbour-Joining method. The results were presented as a dendrograms using UPGMA cluster analysis in NTSYSpc-2.01 program (Rohlf, 1997). The cTBP was performed and analyzed as described earlier (Breviario *et al*., 2007)

Total lipid and FAMES and phorbol ester analysis.

Up to 25 g of *J. curcas* seed kernels were ground using a homogenizer. Total lipid was obtained from powdered seed kernels by soxhlet extraction using hexane as a solvent. Oil recovery was calculated from ratio of oil weight to total seed weight. To a 100 µl oil sample, 1 ml methanol and 1 ml 30 % KOH were added. The samples were then incubated at 75°C overnight. After cooling to room temperature samples were extracted with 1 ml diethyl ether three times. The combined ether extract was dried under a stream of nitrogen. Dried samples were mixed with 1 ml dichloromethane, 1 ml phase transfer catalyst solution (0.1 M tetrabutylammonium hydrogen sulphate in 0.2 M aqueous NaOH) and 25 µl iodomethane using horizontal shaker at 100 rpm for 30 min and then left at room temperature until two phases of the mixtures were separated. The lower phase was transferred to a new tube and then dried under a stream of nitrogen at 40°C. Dried samples were dissolved in hexane and stored at -20°C. *Oil properties.* Saponification number (SN), iodine value (IV), cetane number (CN), and gross energy (GE) were calculated from fatty acid methyl ester compositions of oil using the following equations 1 – 4 (Krisnangkura, 1986; Freeman & Bagby, 1989; Kalayasiri *et al*., 1996)

$$SN = \sum (560 \times A_i)/MW_i \quad : \quad (1)$$

$$IV = \sum (254 \times D \times A_i)/MW_i \quad : \quad (2)$$

$$CN = 46.3 + 5458/SN - 0{:}225 \times IV \quad : \quad (3)$$

$$GE = 618000/SN - 0.08 \times IV - 430 \quad : \quad (4)$$

A_i is the percentage, D is the number of double bonds and MW_i is the molecular mass of the respective fatty acid (mainly palmitic, stearic, oleic and linoleic acid).

The phorbol ester content in the kernels of *J. curcas* and other species of *Jatropha* was determined as described earlier (Makkar *et al*., 1997).

Gas Chromatography.

The Fatty Acid Methyl Esters (FAMEs) were separated by capillary gas chromatography (Perkin Elmer) through a 25 mm methyl silicone (OV-1) capillary column and a flame ionization detector. The temperature profile was run from 100°C to 210°C at a rate of 4°C/min, then held at 210°C for 15 minutes, then increased to 240°C at the rate of 20°C/min and held for 11 minutes to complete the program. Retention time and peak area of each FAME was recorded using a TRIO computing integrator (Trivector System). Comparing the retention times with those of a standard FAMEs mixture identified individual FAMEs.

Determination of water use efficiency (WUE).

Jatropha plants were established in the field under natural photoperiod in Hyderabad, India with a planting width of 2 x 2 m. All measurements were taken on nine month old plants. CO_2 assimilation rates and transpiration were determined by using a portable infrared gas analyzer (LCpro$^+$, Analytical Development Company, Hoddesdon, England). The average light intensity (PAR) was 1500 $\mu E\ m^{-2}s^{-1}$ and the CO_2 partial pressure was 37.5 Pa. Air flow through the analyzer was adjusted to maintain cuvette relative humidity near ambient levels of 55-65% during measurements. The rates of CO_2 exchange were determined on five plants at regular intervals. The measurements were taken on clear days during three periods: early summer (May, 2007), midsummer (June) and late summer (August). Measurements were taken on fully expanded mature leaf number three counted from the shoot apex. Water use efficiency was calculated as *A/E* (See Table 3).

Results

Genetic diversity in Jatropha

Four accessions (JI, JI-2, JN and JT) from India, Nigeria and Thailand respectively, were supplemented with 13 additional *J. curcas* accessions from six provenances of Thailand and one accession of *J. podagrica,* as an outgroup*,* also from Thailand. These 18 samples were initially examined using 10 RAPD primers. The resultant profiles subjected to UPGMA-mediated cluster analysis revealed two major clusters (Figure SF1). One cluster contained all of the 17 *J. curcas* accessions and the second contained the out-group *J. podagrica*, which showed an overall similarity of 52% with *J. curcas*. Among the *J. curcas* accessions the similarity coefficient was high (0.78) indicating a narrow genetic base. The two Indian accessions clustered separately while the Nigerian accession clustered with the remaining 14 Thai accessions. The 6 provenances of Thai accessions could not be clearly differentiated, reinforcing the narrow genetic base between provenances.

The *J. curcas* accession from Mexico known to bear seeds containing minimal amounts of ANFs and PEs was expected to be genetically diverse. This non-toxic Mexican accession (NTMA) was then investigated together with 6 Thai accessions from 4 provenances using AFLP with 32 primer pairs. Results showed the former to be genetically distant from the Thai accessions (Figure SF2), yet 76% similar, whereas the Thai accessions again turned out to be highly similar between themselves (90%). These results indicated the importance of a) testing accessions from wider eco-geographic regions; b) assessing other *Jatropha* species in addition to *J. curcas,* and c) assessing natural and artificial interspecific hybrids for genetic variability.

We thus investigated the following material: a set of *J. curcas* accessions obtained from various parts of the world, including NTMA; different species of *Jatropha* obtained from India; a natural hybrid

occurring in India (Prabakaran & Sujatha, 1999); synthetic hybrids and a somaclonal variant generated earlier in India (Sujatha & Prabakaran, 2003) and additional hybrids generated for this study between *J. curcas* and *J. maheshwarii*/*J. multifida* (Please refer to Table ST1 for details on different accessions and species used). A novel, relatively unexploited technique of combinatorial tubulin based polymorphism (cTBP; Breviario *et al*., 2007) was used. The method uses variation in the length of the first and second intron of members of the plant β-tubulin gene family. The approach was successfully used earlier to detect intra and inter-species polymorphism in diverse plants including oilseed plants - rapeseed and peanut (Breviario *et al*., 2007) and palm (Breviario *et al.,* 2008). Two sets of cTBP were performed on *J. curcas* accessions from different parts of the world. In the first set cTBP analysis on 10 *J. curcas* accessions from 9 different countries in Asia, Africa and the Americas revealed no polymorphism for either intron (Figure SF3). Although this was surprising in that nearly 10 different plant genera and their respective species and accessions put through cTBP analysis never exhibited monomorphism earlier (DB - unpublished results), it was not surprising in view of the RAPD and AFLP results obtained with *J. curcas* earlier. In the second set 16 accessions from 6 countries were analyzed. Whereas some accessions in the second set were identical to the first set, those from Tanzania, Surinam and Costa Rica were new. Results showed that the four accessions from Costa Rica were clearly different to those from other parts of the world and they also exhibited intra-specific polymorphism in both intron I and II (Figure SF4). The ability of cTBP to differentiate between accessions was then used in the following cTBP analysis to differentiate between species. The NTMA which revealed polymorphism by AFLP analysis earlier was tested together with some other *J. curcas* accessions; different *Jatropha* species; natural and synthetic hybrids of *Jatropha* and a somaclonal variant of *J. integerrima*. These investigations showed that the NTMA clustered separately from the other *J. curcas* accessions and indeed the different species of *Jatropha* exhibited marked genetic variability (Figure 1). These results demonstrate the value of cTBP analysis in fingerprinting the *Jatropha* genus. UPGMA analysis of

the cTBP bands gave genetic similarity coefficients over the range of 0.07 to 1.0 (Figure 1), with three of the four *J. curcas* accessions typically showing a high similarity amongst them and with NTMA. Other species were highly dissimilar. As expected the 2 accessions of *J. integerrima* that differed in flower colour and the somaclonal variant were markedly similar to each other but also to *J. rosea*.

The cTBP analysis was again used to assess the genetic variability in the F_1 generation compared to the parental species for hybrids of *J. curcas* with *J. integerrima, J. maheshwarii* or *J. multifida* (Figure 2). Clear polymorphism was indicated between the parental types and the resulting F_1 progeny plants with facile identification of parental bands in the hybrids. The 1:1 inherited band pattern of the F_1 hybrid with respect to parental contribution indicates the homozygous status of the parental lines that may not be restricted just to the alleles of the beta-tubulin gene family. Our results reiterate the value of cTBP as a genotyping tool for plant accessions, species and hybrids. The method is simple and rapid and can be applied to any plant genome of unknown sequence.

Seed phorbol ester content

Table 1a shows that *J. curcas* accessions and species have a wide range of PE content with the NTMA showing undetectable to very low amounts of PE in the seed. The material for a potentially successful conventional breeding approach is thus available in nature. An earlier attempt at trying to obtain low PE plants after crossing the NTMA with standard *J. curcas* was not successful (Sujatha *et al*., 2005), most likely due to a limited genetic variability. To circumvent this limitation interspecific hybrids generated earlier (Sujatha & Prabakaran, 2003) and for this study were analysed. We compared the F_1 population of interspecific reciprocal hybrids between *J. curcas* as one parent and *J. integerrima, J. maheshwarii or J. multifida* as the other parent for yield and desirable agronomic traits including the reduction in PE content in glasshouse experiments. We obtained one line that

exhibited comparatively better agronomic traits and low PE. This line was backcrossed to *J. curcas* NTMA accession in order to further reduce the PE content. Indeed, we obtained two BC lines (ID 222 and 223) with PE levels comparable to those in NTMA (Table 1b). Along with reduced PE these lines exhibited quantitatively and qualitatively improved seed as seen through the number of fruits per inflorescence (23-25), seed weight, oil content, oleic acid to linoleic acid (O/L) ratio and PE level (Table 2). This indicated the potential for breeding elite *Jatropha* cultivars. These data suggest that the chances of improvements in commercially desirable traits of *J. curcas* are higher through interspecific breeding rather than the intraspecific breeding approach, which is limited by the low genetic variability in globally distributed accessions of *J. curcas*.

Phenotypic variability: seed size, oil content and composition and water use efficiency

High genetic similarity in *J. curcas* seeds of accessions collected from different countries and provenances suggested that seed phenotype parameters such as seed size and percent kernel weight might not be significantly different in. However, our data confirmed high variability in seed phenotypic characteristics (Table ST2a). For example, standard deviation (SD) and sample variance (SV) in mean seed weight (n=10) of seeds from 7 different eco-geographic regions in Table S1a was 0.1019 and 0.0102 respectively. However, SD and SV within the 10 seeds of each region varied from 0.017 to 0.022 and 0.00061 to 0.00073 respectively. This indicated higher variability between the regions than within the regions, in turn indicating that despite high genetic similarity there was high phenotypic variability in seed weight, most likely due to differences in the respective environmental and eco-geographical conditions.

Boschin *et al*. (2008) recently demonstrated that in oilseed lupin when a total of six cultivars from Spain, France and Italy were assessed in three different climates, the variation in total oil content and fatty acid composition was more dependent on the genotype than on genotype environment (GE)

interaction This suggested limited variability in these traits among the *J. curcas* accessions exhibiting high genetic similarity. However, the variation in total oil content of *J. curcas* seeds from different regions (Table ST2b) exhibited a high SD and SV of 7.5 and 56.6 respectively suggesting environment-mediated changes in oil content. In parallel, since the biodiesel quality depends on high O/L ratio, we analysed the O/L ratio of different accessions coming from different eco-geographic regions The O/L ratio (Table ST2b) also exhibited high SD and SV (0.25 and 0.06 respectively) compared to a low SD and SV (0.094 and 0.008 respectively) within 10 different samples from the same field in the case of accession JI, once again implicating environmental variables for the noticed differences. Saleem *et al*., (2008) demonstrated that in sunflower, oil composition was affected by environmental variables of temperature and humidity arising due to different sowing times.

The Δ-12 fatty acid desaturase (FAD) is responsible for conversion of oleic acid to linoleic acid contributing to the final O/L ratio in the seed oil. We used semi-quantitative RT-PCR for the *fad* gene on a small set of three *J. curcas* accessions (JI, JN and JT), to see if a relationship existed between the O/L ratio and the *fad* transcript level. As evident from Table S2b, JT was chosen due to its lowest O/L ratio and JI and JN were chosen for being at the higher end of the O/L ratio values amongst the ten accessions. The RT-PCR analysis showed 1.6 times more *fad* transcript in JT than in JI or JN (Figure 3), suggesting a higher amount of FAD function leading to increased conversion of oleic acid to linoleic acid and in turn leading to the low O/L ratio observed. A higher amount of *fad* transcript accumulation in JT seeds is more likely due to epigenetic rather than genetic reasons as discussed below.

Water use efficiency (WUE) in a drought tolerant plant such as *J. curcas* is an important physiological trait. We assessed four different Indian accessions of *J. curcas* for WUE under identical environmental conditions in the field. One of these; TCR193 clearly exhibited a relatively

high WUE (Table 3). However, Table 3 also shows that differences in WUE between the four accessions were mainly because of differences in CO_2 fixation efficiency rather than to differences in transpiration rate, which were largely similar. Such relatively large differences in the photosynthetic rate under identical environmental conditions implicate strong genetic determinants. Since these accessions were not compared for genetic variability, it was not clear if differences in the photosynthetic rate had a genetic base. Therefore, we compared the WUE of JI – one of the accessions used in the field study – to two other accessions JN and JT, in a controlled environment chamber. JI, JN and JT were genetically more than 80% similar (Figures SF1 and 1). The dissimilarities are not necessarily expected in essential (hence largely conserved) genes such as the ones involved in photosynthesis. Hence, photosynthetic rate, transpiration rate and the WUE were expected to be largely similar. However, once again as in the field study, we noticed similar transpiration rates and WUE values but relatively large differences in photosynthetic rates under identical growth conditions. These results implicate epigenetic imprint-mediated expression differences in otherwise similar genomes.

Our results for variability in seed phenotype and FA content and composition suggest epigenetics-mediated adaptation and those with WUE suggest such adaptations being passed to the next generation. Recent studies do indicate that ecologically adaptive epigenetic variation in natural populations can be independent from genetic variation, and that such environmentally induced epigenetic changes are inherited by future generations in some cases (Bossdorf *et al*., 2007).

Discussion

The *Jatropha* plant is a new system and only recently exposed to molecular investigations, mainly due to its increasing popularity as a biodiesel feedstock and valuable co-products (Kohli *et al*.,

2009). The few studies on genotyping of *J. curcas* are based on assessing Indian accessions. Reddy *et al.* (2007) used AFLP/RAPD on 20 Indian accessions while Basha & Sujatha (2007) used 400 RAPD and 100 ISSR primers on 42 accessions from different regions in India. They also included the NTMA in their analysis and although it could be easily differentiated from the Indian accessions, there was 70% similarity between the two. Ranade *et al*., (2008) used single-primer amplification reaction (SPAR) to compare 21 accessions from different parts of India and demonstrated that 3 North East accessions were different among them and from other accessions analysed. Ram *et al*., (2007) and Pamidiamarri *et al*., (2008) compared *J. curcas* with additional *Jatropha* species from India and demonstrated clear divergence. In all these studies *J. curcas* accessions from different eco-geographic regions of India were 60 to 80% similar. Results from the studies above suggested the importance of testing accessions from wider eco-geographic regions of the world. However, our analysis of 38 accessions from 13 countries around the world, along with 6 different species of *Jatropha* from India, again indicated 75% similarity among the global *J. curcas* accessions. We also showed that the NTMA clustered separately from other *J. curcas* accessions and that the genetic similarity coefficient between the Thai and the NTMA was high (0.76) as similarly noted by Basha & Sujatha (2007). Our assessment of nearly 52% similarity between the Thai *J. curcas* and *J. podagrica*, was also the same as reported by Ram *et al*., (2007) in the case of Indian *J. curcas* and *J. podagrica*. Our comparison of global accessions of *J. curcas* to other *Jatropha* species resulted in *J. integerrima* and *J. gossypifolia* being closer to *J. curcas* than other species as noted by Pamidiamarri *et al*., (2008) in comparing the Indian *J. curcas* to other *Jatropha* species from India. Such an overlap between local and global results indicates a narrow genetic diversity in *Jatropha*. Incidentally, preliminary data suggests that *J. curcas* accessions exhibit monomorphism even with microsatellite markers (Drs. Gen Hua Yue and Hong Yan, Temasek Lifescience Laboratories, Singapore - personal communication), indicating very low genetic divergence among accessions spread around the globe. The reasons for the globally low genetic variability seen in *J. curcas* are not

clear. Most likely, the anthropogenic and environmental influences in generating genetic variability are missing because a) it is not a crop, b) as a well-surviving, undomesticated plant, it is highly stress tolerant due to adaptive genomic characters probably acquired before its global distribution and c) a limited stock has been vegetatively and apomictically propagated, since *J. curcas* is known to exhibit apomixis (Bhattacharya *et al*., 2005).

The studies mentioned above indicate the limitations of using intra-specific breeding for *J. curcas* improvement; as exemplified by the failure to obtain low PE hybrids in crosses between *J. curcas* toxic accessions from India and the NTMA (Sujatha *et al.*, 2005). Hence we tried the interspecific hybridization approach. Of the three out-species we used (*J. integerrima, J. multifida* and *J. maheshwarii*) the hybrids that survived the best were those of *J. curcas* x *J. integerrima*, reiterating the relatedness between the two species. *J. integerrima* does not exhibit most agronomic or commercially useful traits to desirable degrees, for example it contains the highest amount of seed PE, second only to *J. multifida* (Table 1a). It is also a plant with very thin stem, an intermediate level of oil content and a low O/L ratio (Table ST2c) in contrast to the high value desirable. However, analysis of the F_1 plants revealed advantages gained through hybridisation, in agronomic and commercial traits (Table 2) including low PE levels (Table 1b). A back cross with *J. curcas* NTMA further reduced the PE levels comparable to NTMA – a highly desirable target (Table 1). Our analysis revealed higher genetic distance (GD) between *J. curcas* and *J. integerrima* than between *J. curcas* accessions. Higher GD favourably affects F_1 performance (Kiula *et al*., 2008). Interspecific hybridization with compatible species leads to generation of valuable material due to heterozygosity at several loci. The affect could be more pronounced if most loci in the parents were homozygous to start with. Our cTBP analysis of the hybrids (Figure 2) indeed suggests parental lines homozygous for alleles of the beta-tubulin gene family – one that is known to be variable (Breviario *et al*., 2007; 2008), in turn suggesting the possibility of homozygosity at the other loci. Additionally, a larger

numbers of genes express differential alleles in a hybrid (Guo *et al*., 2008), which if resulting from genetically diverse parents permits larger numbers of permutations and combinations, in turn resulting in pronounced range of differences in the progeny in comparison to the parents. A combination of such possibilities may underlie our observations of improved hybrids despite *J. integerrima* apparently not being an ideal material. Our preliminary data with selection of plants from additional back cross progeny (BC_3) exhibits stable inheritance of the improved characters illustrated in Table 2. In any case, our results suggest that excluding the Indian accessions due to their narrow genetic base and trying to find sufficient diversity within *J. curcas* around the world for selecting breeding lines may have very limited value due to the globally narrow genetic base of *J. curcas* that we illustrate.

There are a few reports on genetic characterisation of *J. curcas* and even fewer on systematic assessment of phenotypic characteristics of different accessions. We noticed a large variation in seed size and oil content in seeds from various parts of the world, while they exhibited high genetic similarity. Kaushik *et al*., (2007) reported the predominant role of the environment in the higher phenotypic coefficient of variation in some characters in 24 Indian accessions *J. curcas* originating from different agroclimatic zones. Although they did not conduct genetic similarity tests, our results and other genotyping studies mentioned above suggest a high likelihood of a narrow genetic base in the accessions tested. Our results of large differences in morphological and biochemical phenotype for seed size and seed oil content and composition respectively, in accessions with a narrow genetic base, implicated environmental factors-mediated changes. Recently, Sunil *et al*., (2008) recorded the phenotypic traits of *J. curcas* plants *in-situ* at 4 different eco-geographical regions of India. They noticed pronounced differences in the 9 characters they assessed for a total of 162 accessions in the 4 zones. For example, the plant height of 80% accessions in one zone was less than 1.5 meters while in another zone 60% of the accessions were larger than 1.5 meters. Similar differences were noticed in

number of fruits and seed oil content and composition. Environment related developmental differences noticed in *J. curcas* by Sunil *et al*., (2008) are reminiscent of such differences noticed in *Achillea* in the classical study by Clausen *et al*., (1958) wherein an identical genotype (clonal cutting) grew to be the tallest at low elevations but was the shortest plant at medium elevations and the second tallest at high elevations. Since Sunil *et al*., (2008) also did not undertake genetic characterisation of the accessions, the reason for the variability was not clear. However, once again, considering our results and the genetic studies conducted in India and mentioned above, it is not unreasonable to expect a narrow genetic base in the 162 accessions tested. If highly related Indian accessions demonstrated large phenotypic variability then in our study, similarly related accessions from totally different countries are prone to showing even larger phenetic variability.

Our analysis of WUE in *J. curcas* is the first such report to the best of our knowledge. Although our WUE results provide only a general idea under a single set of conditions, both in the field and in the CER, we obtained reproducible WUE values in the field in India and in the CER in the UK, which compared well with WUE studies on other hardy plants. For example, the range of WUE of olive trees, a hardy plant like the *J. curcas*, in Tunisia on marginal rain-fed sandy soil and under similar but irrigated land varied between 4.7 to 7.6 mmol/mol (Ben-Rouina *et al*., 2007). Similar transpiration rates in different accessions of *J. curcas* may indicate similar stomatal density. Since the three accessions tested in CER originated from three countries, differences in photosynthetic rates and hence in WUE in genetically highly similar accessions under similar environmental conditions implicate allopatric, epigenetic and stochastic factors.

One character that has been well studied for environment dependent changes in plants is flowering in plants such as *Arabidopsis and Antirrhinum*. In the case of *Jatropha,* flowering phenology was studied for an accession from Cape Verde on a field in Nicaragua (Aker, 1997). Flowering time,

number and male:female flower ratio all varied substantially depending on soil fertility, soil moisture, precipitation, Piche evaporation and temperature. Our unpublished observations are similar to environment-dependent differences noticed by Aker (1997) in that a *J. curcas* accession exhibits one major flowering flush in one eco-geographical zone (around Hyderabad in India) whereas in another zone (the more humid parts of Kerala in India) it exhibits episodic flowering depending on rain and moisture conditions.

A pronounced phenotypic plasticity is in itself a genotypic trait that allows the plant to respond to different environments through morphological and physiological changes for its survival (Richards *et al*., 2006). In our study, although genetic analysis clustered accessions from a single region separately (Figures SF1 and SF2), interestingly, clustering the accessions based on the data in Table ST2 indicated similarity between accessions depending on the origin of the seeds, highlighting the influence of the local conditions. For example, accessions from particular provenances of Thailand clustered together through such an analysis (Figure SF5). We propose that high genetic similarity in global accessions of *J. curcas* can exhibit large phenotypic variability largely through epigenetic mechanisms. The potential for epigenetic marks to contribute to heritable differences depending on environmental differences has been recently examined (Bossdorf *et al*., 2008; Grant-Downton & Dickinson, 2005) including in similar seed traits of *Arabidopsis* (Blodner *et al*., 2007) as studied for *J. curcas* in this report. The molecular basis of phenetic variability in genetically similar backgrounds may likely reside in stochastic and/or epigenetic factors regulating gene transcription. We noticed a relatively higher amount of the *fad* gene transcript, most likely responsible for the low O/L ratio in JT seeds, as compared to that in JI and JN seeds. Similarly, JI exhibited larger amounts of seed triacylglyceride (TAG) and revealed larger amount of the diacylglyceride acyl transferase (*dgat)* transcript (data not shown). Given the general genetic similarity, it is unlikely that the difference we noticed in the transcript level of essential genes such as *fad* and *dgat* in JT and JI

respectively were due to differences in the respective promoters of the genes. Blodner *et al.,* (2007) recently discussed the temperature-based differential transcription of fatty acid desturases in other oilseed plants. We have cloned and compared the coding region and promoter of the phosphoenol pyruvate carboxylase (*pepc*) gene and three oleosin genes from JI and JN and found them to be identical. Transcript-based expression analysis of these genes exhibit differences similar to the *fad* gene (manuscript under preparation). Moreover, the lack of polymorphism resulting after AFLP analysis of *J. curcas* has been resolved in favour of an epigenetics-based hypothesis due to a high degree of polymorphism noticed with methylation-sensitive AFLP, implicating differential methylation of genes in various accessions (Dr. Hong Yan, Temasek Lifescience Laboratory Singapore – personal communication). Finally, phenetic variability in the face of widespread homozygous loci as preliminarily hypothesised through our cTBP results on parental and F_1 hybrids (Figure 2) adds to the proposition of epigenetics-mediated reasons for the changes noticed and suggests the strength of proteomic-based approach.

For *J. curcas* to be a commercially viable source of biodiesel much research and development is needed and little should be expected from the present widespread planting of wild varieties. Sujatha *et al*., (2008) recently reviewed the role of biotechnology in the improvement of *J. curcas.* A combination of breeding and transgenic approaches could deliver elite varieties. Whether or not this happens depends very much on global commodity markets and the competition from other energy technologies and energy crops. However, the simultaneous value of *J. curcas* as a novel research system must be appreciated. Its difference in habit as a shrub compared to model herb *Arabidopsis* and model tree poplar may be important in terms of the extent of DNA and histone modifications; just as such difference exists between *Arabidopsis* and poplar.

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Supporting Information:

We include 5 Figures: SF1 – SF5 and 2 Tables: ST1 – ST2 as supporting information and the context in which they must be referred to is mentioned in the main text of the manuscript

Figure legends

Figure 1

UPGMA of cTBP analysis on different species of *Jatropha*. *Jatropha* species were clearly different from each other and from *J. curcas* accessions which were often closely related.

Figures 2

cTBP analysis showing polymorphism in intron I between the parental and hybrid plants for I: *J. curcas* X *J. integerrima;* lanes 1, 2, 3, 4, 5 and 6: J.c, hybrid 1, hybrid 2, hybrid 3, hybrid 4 and J.i respectively; II: *J. curcas* X *J. gossypifolia = J. tanjorensis;* lanes 7, 8 and 9: J.g, J.t and J.c respectively; III: *J. curcas* X *J. multifida*; lanes 10, 11 and 12: J.mu, hybrid and J.c respectively; IV: *J. curcas* X J. *maheshwarii* lanes 13, 14 and 15: J.ma , hybrid, J.c respectively; M: DNA size marker)

Figure 3

Gas chromatographic analysis of seed fatty acid profiles of accessions JI, JN and JT showing higher amounts of linoleic acid than oleic acid in JT, also reflected in the larger amount of the *fad* transcript (which converts oleic to linoleic acid; see inset) in JT.

Supporting Figures

Figure SF1

UPGMA analysis of RAPD conducted on 17 *J. curcas* and one *J. podagrica* accession. Origin of accessions identified as follows: JI & JI-2 – India; JN – Nigeria; JT – Thailand. All other accessions

were from different provenances of Thailand represented here as TP. ST – TP1; KU – TP2; CB – TP3; KS – TP4; PB – TP5; PC8, 18, 29, 32, 62, 69, 76, 81 – TP6 and JPO – Thailand *J. podagrica.*

Figure SF2

A. Representative gel showing DNA amplicons obtained using two separate AFLP primer sets (I and II) on six *J. curcas* accessions (lane 1 to 6) from Thailand and one (lane 7) from Mexico (Non-toxic; NTMA). M: DNA size marker. Lack of polymorphism in the Thai accessions was representative of similar results obtained with additional primer sets and other *J. curcas* accessions from India and Nigeria. The polymorphic bands (present: blue circles; absent: yellow circles) in NTMA indicate its genotypic diversity in comparison to the Thai (and Indian and Nigerian) accessions analysed. B UPGMA analysis of the AFLP results with the 32 primer pairs. Numbers represent the following accessions: 1:Chaing Mai 1, 2: Chiang Rai 1, 3: Packchong 6, 4: Packchong 73, 5: Korat 1, 6: Packchong 43, 7: NTMA. The analysis revealed high similarity between the Thai accessions and the NTMA and a higher similarity between the accessions from Thailand.

Figure SF3

Combinatorial Tubulin Based Polymorphism (cTBP) for β-tubulin gene family introns. Representative gel for cTBP on 10 accessions from 9 countries (1: Cape Verde; 2: Egypt; 3: Ecuador; 4: India (JI); 5: Indonesia; 6: Nigeria (JN); 7: Thailand (JT); 8: Mali; 9: Nigeria; and 10: Paraguay. M: DNA size marker). Gel shows a distinct lack of polymorphism in both intron I and II.

Figure SF4

cTBP analysis for intron I (a) and II (b) for 16 *J. curcas* accessions from 6 countries - Tanzania (1 to 4 and 14); Surinam (5 to 8); Costa Rica (9 to 12); India (13); Egypt (15) and Thailand (16). M: DNA size marker. Accessions from Costa Rica were clearly different within and between the countries.

Figure SF5

Measured values for shaded properties in Table ST2 were computed to assess the accession with an optimal trait combination (oil*O/L*CN/Kernel weight*%Kernel). SF5a shows that accession JN and JI were superior. However, low PE content in JI makes it an even better candidate. SF5b shows the relationship between the accessions based on the standardized data set [(x-meanx)/Sdx]. Relatedness reflected the eco-geographical influence on seed properties on a set of accessions highly similar at genotypic level. The 6 provenances of the Thai accessions were clearly separated which was not the case through genotypic clustering (see main text).

Figure 1. UPGMA analysis of cTBP conducted on various accessions and species of *J. curcas*

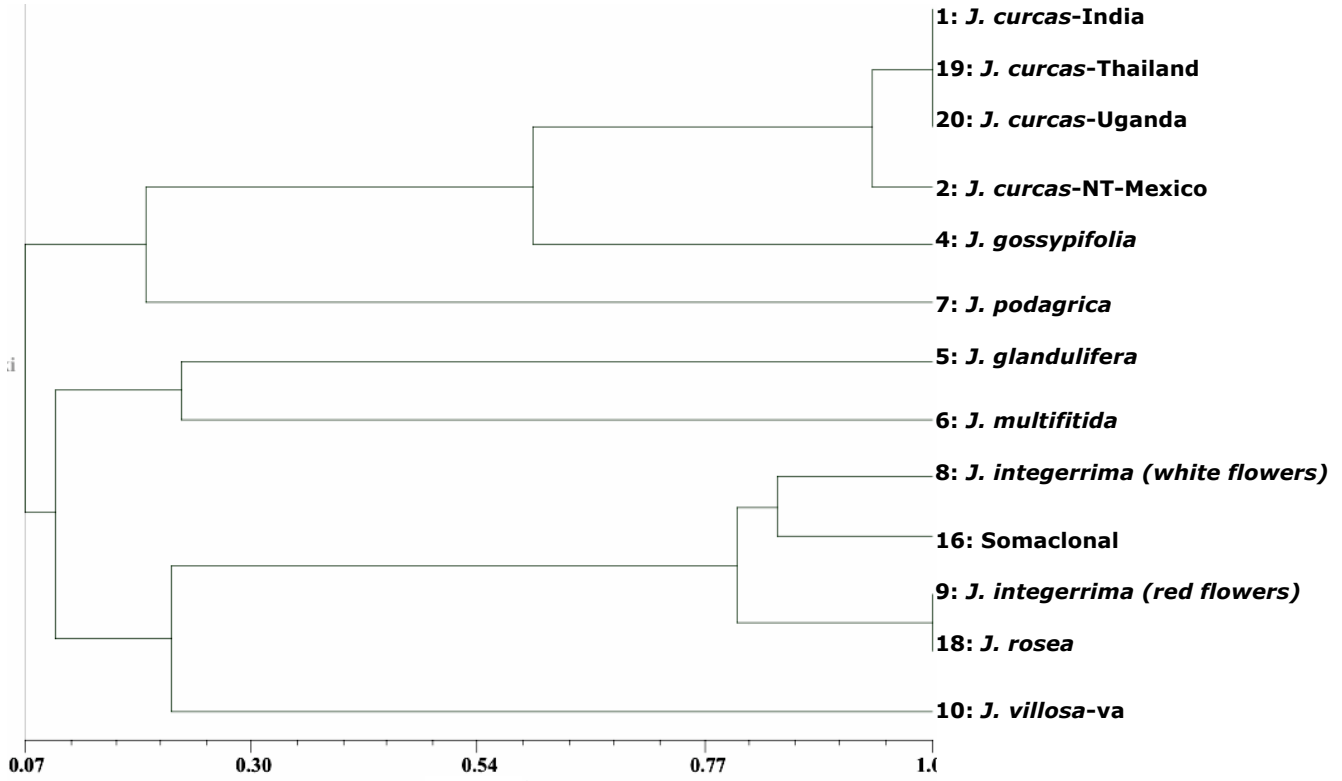

Figure 2. cTBP-mediated genotyping of the inter-specific hybrids and parents

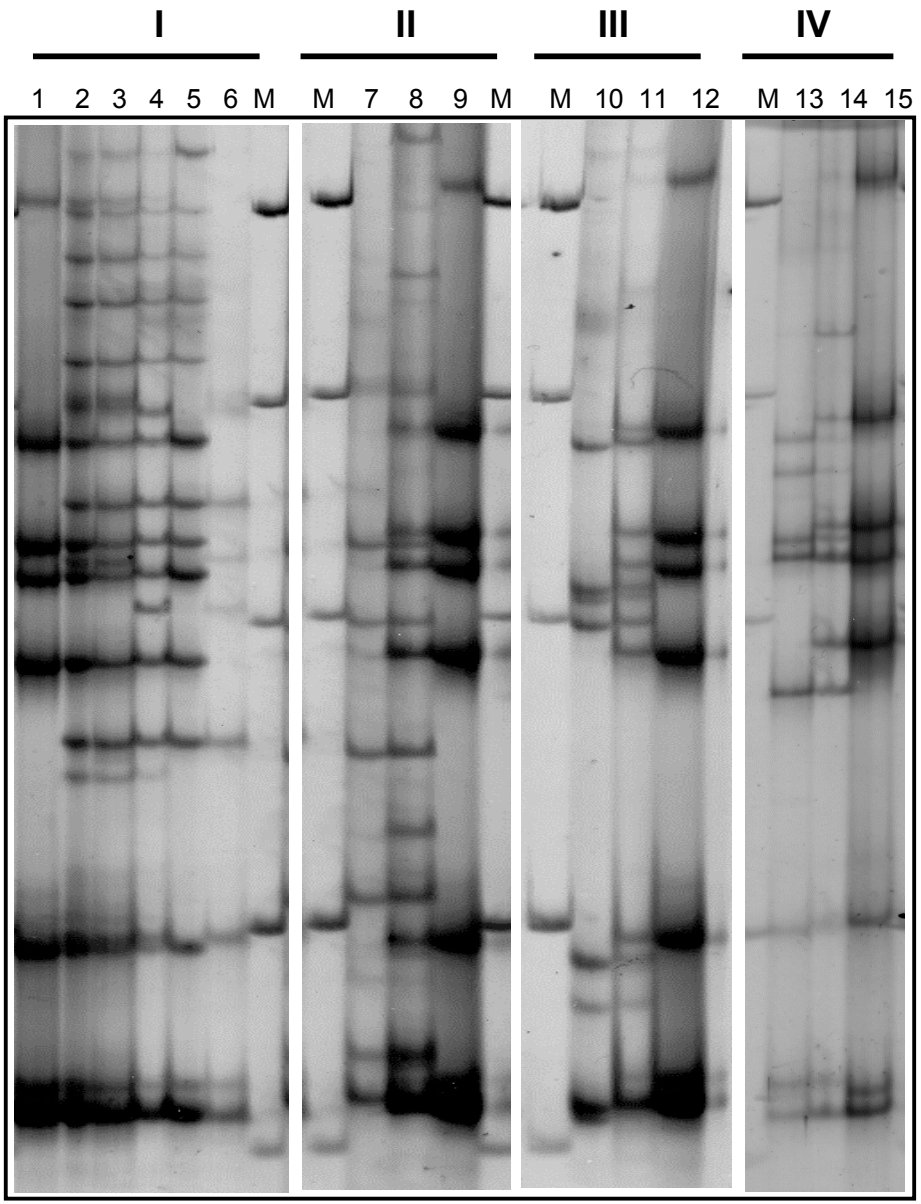

Figure 3. Relationship between FA biosynthesis gene transcripts and FA profile in the seeds of *J. curcas*

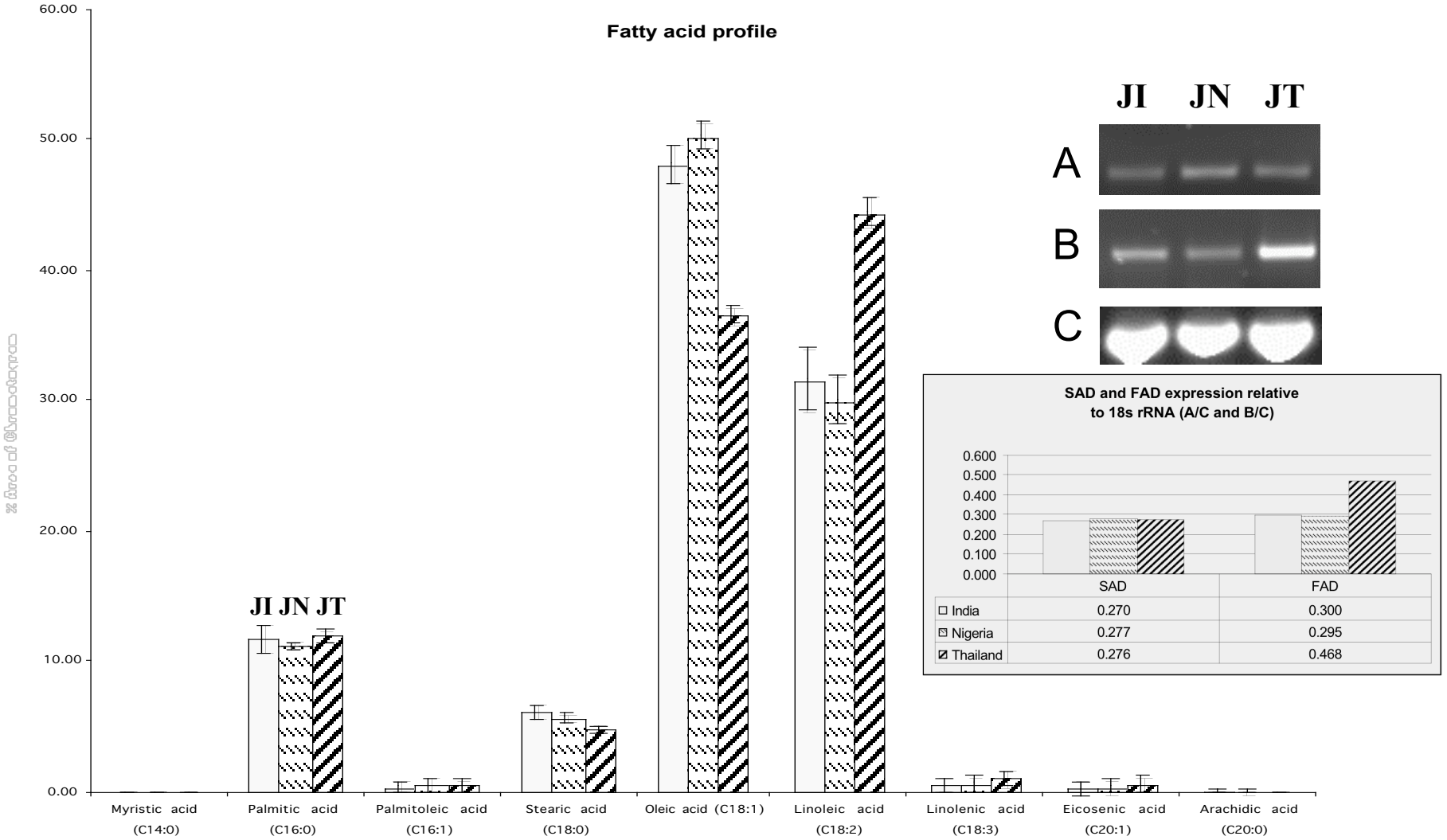

Table 1a. Phorbol Ester content in *Jatropha* kernels

Species	Sample	PE (mg gm-1 kernel)
J. curcas	NTMA*	ND** - 0.11
J. curcas	JI	1.62
J. curcas	IJC-4	2.22
J. curcas	IJC-1	2.62
J. curcas	IJC-2	2.65
J. curcas	IJC-11	3.81
J. curcas	Kerala	3.9
J. curcas	JI-1	4.12
J. curcas	JI-2	4.3
J. curcas	Chintapally	5.04
J. integerrima	IJI	7.92
J. glandulifera	IJGL	2.78
J. podagrica	IJP	4.5
J. multifitida.	IJM	9.09

Table 1b. Phorbol Ester content in *Jatropha* hybrid kernels

Hybrid (Jc.Jint)	ID 222	0.08
Hybrid (Jc.Jint)	ID 223	0.12
Hybrid (Jc.Jint)	ID 50	1.71
Hybrid (Jc.Jint)	IH 112	2.2
Hybrid (Jc.Jint)	ID 111	2.52
Hybrid (Jc.Jint)	ID 93	2.8
Hybrid (Jc.Jint)	ID 225	2.82
Hybrid (Jc.Jint)	ID 226	3.31
Hybrid (Jc.Jint)	IH 13	4.1
Hybrid (Jc.Jint)	IH 300	4.43

Hybrid, analysis of seeds on *J. curcas* obtained after pollination by pollen of F1 flowers of *J. curcas* (f) x *J. integerrima* (m) cross. Each sample represents an independent back cross (BC1)
*NTMA: Non-Toxic Mexican accession. **ND = Not Detected

Table 2. Comparative range of critical seed properties for interspecific derivatives and *Jatropha curcas*

Trait	**Interspecific**		***J. curcas***	
	n	Range	n	Range
Seed weight (g)	19	0.23-0.78	5	0.22-0.77
Seed oil content (%)	14	16.39-34.47	4	29.8-33.58
Oleic (%)	22	24.92-52.95	6	36.28-51.18
Linoleic (%)	22	25.66-44.89	6	27.27-60.92
Phorbol esters (mg/g)	12	0.04-9.04	8	0.01-5.25

Table 3a. Differential Water Use Efficiency (WUE) of*Jatropha curcas*plants in the field.

Species	**Sample**	**Photosynthetic rate** (A mmol m-2s-1)	**Transpiration rate** (E mmol m-2s-1)	**Water use efficiency (WUE)** (A/E mmol/mol)
J. curcas	JI	30.12	6.03	4.99
J. curcas	TCR31	28.26	5.87	4.81
J. curcas	TCR193	35.38	5.05	7.01
J. curcas	TNC-10	22.46	5.85	3.84

Table 3b. Differential Water Use Efficiency (WUE) of*Jatropha curcas*plants in the CER.

Species	**Sample**	**Photosynthetic rate** (A mmol m-2s-1)	**Transpiration rate** (E mmol m-2s-1)	**Water use efficiency (WUE)** (A/E mmol/mol)
J. curcas	JI	31.12	7.03	4.42
J. curcas	JT	28.21	7.91	3.57
J. curcas	JN	37.31	7.85	4.75

Figure SF1. UPGMA analysis of RAPDs conducted on accessions of *J. curcas*

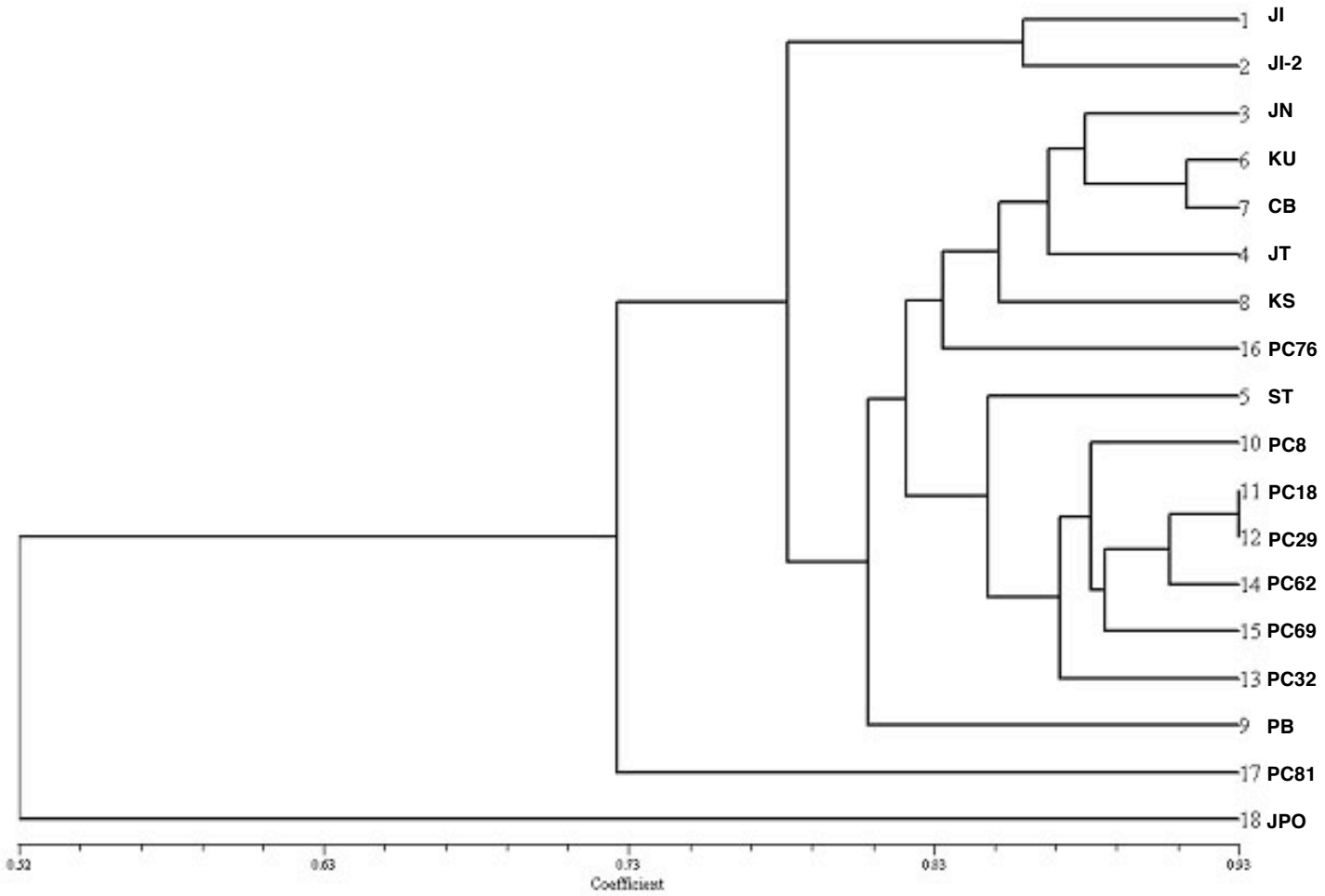

Figure SF2a. AFLP-mediated differences between the NTMA and 6 Thai accessions of *J. curcas*

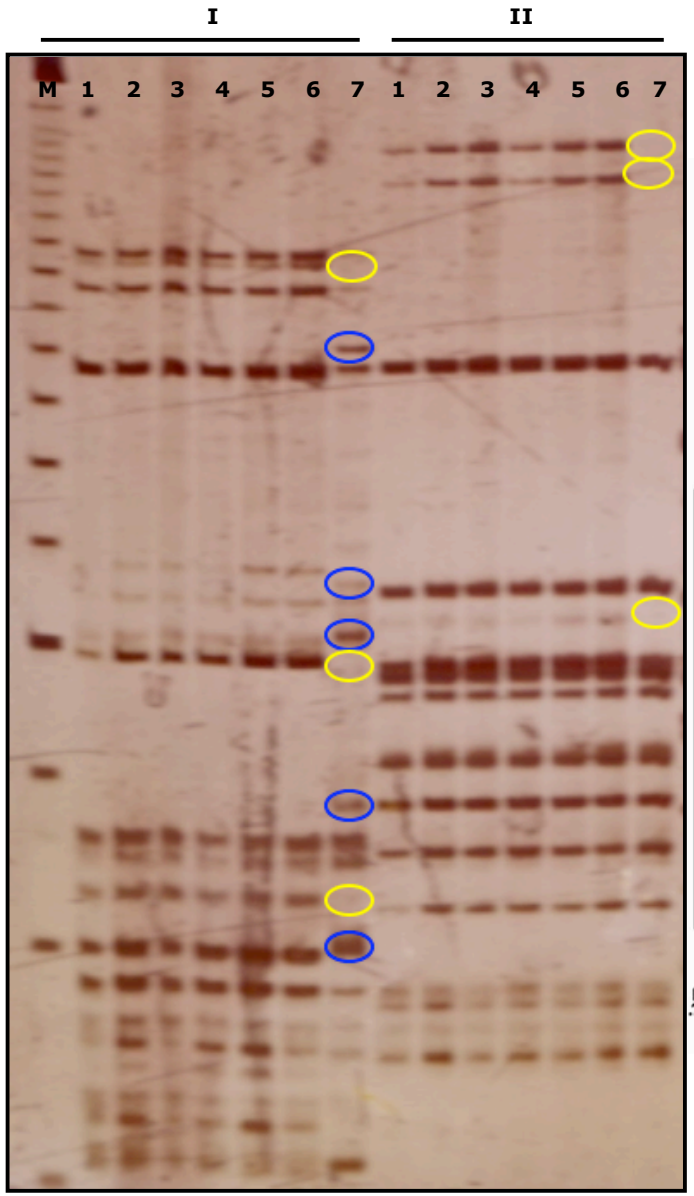

Figure SF2b. UPGMA analysis of AFLP conducted on NTMA and 6 Thai accessions of *J. curcas*

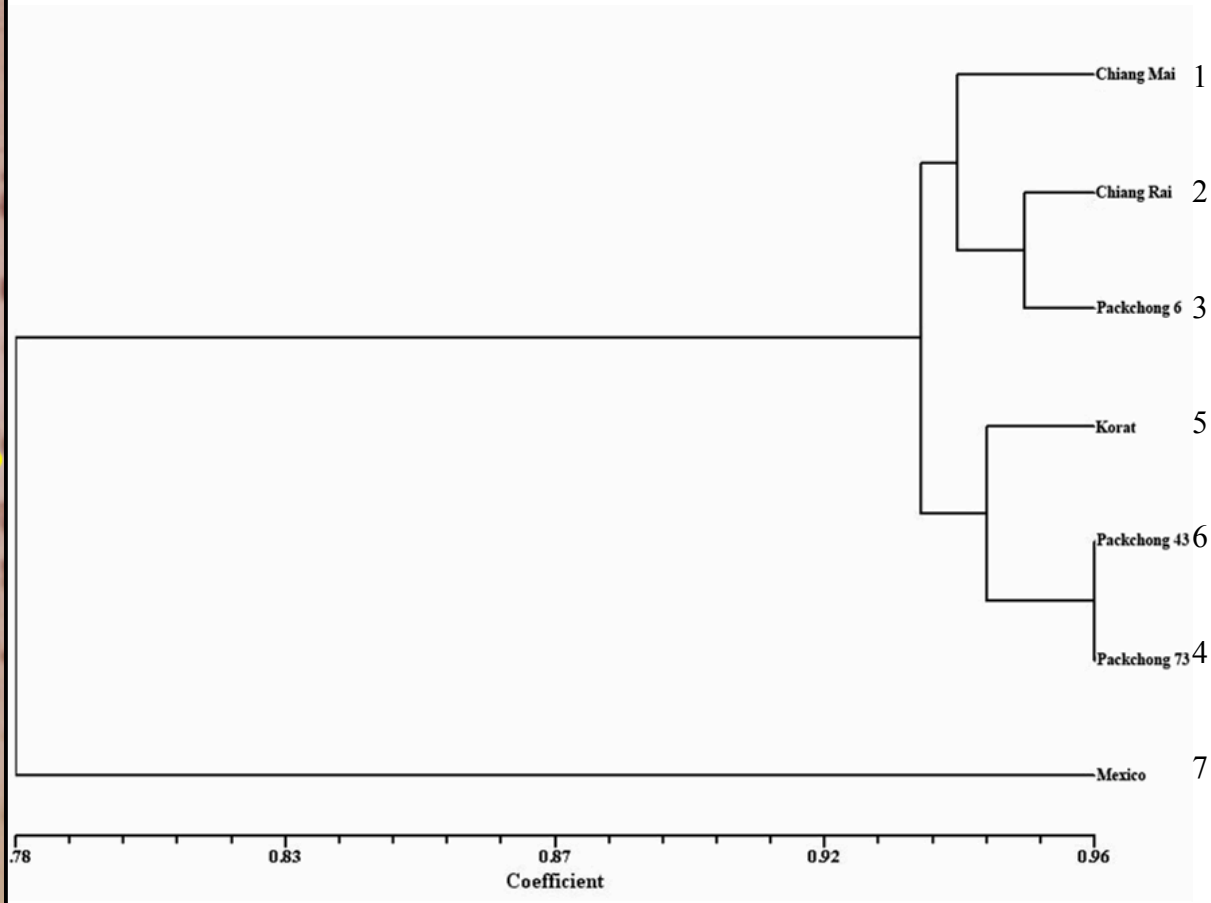

Figure SF3. cTBP-mediated detection of monomorphism in *J. curcas*

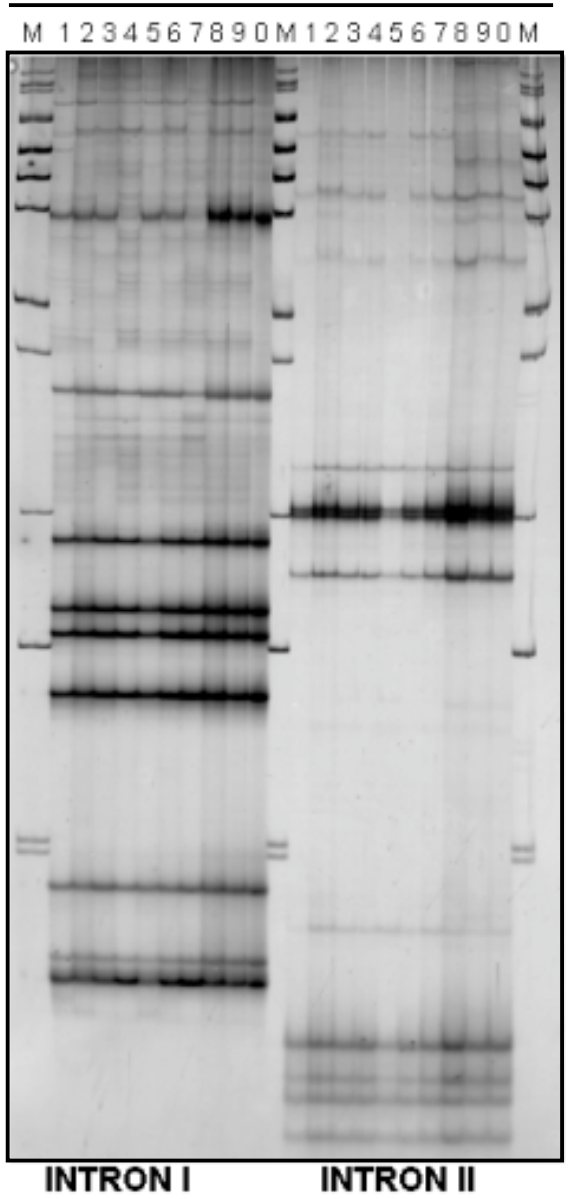

Figure SF4. cTBP-mediated genotyping of *J. curcas*

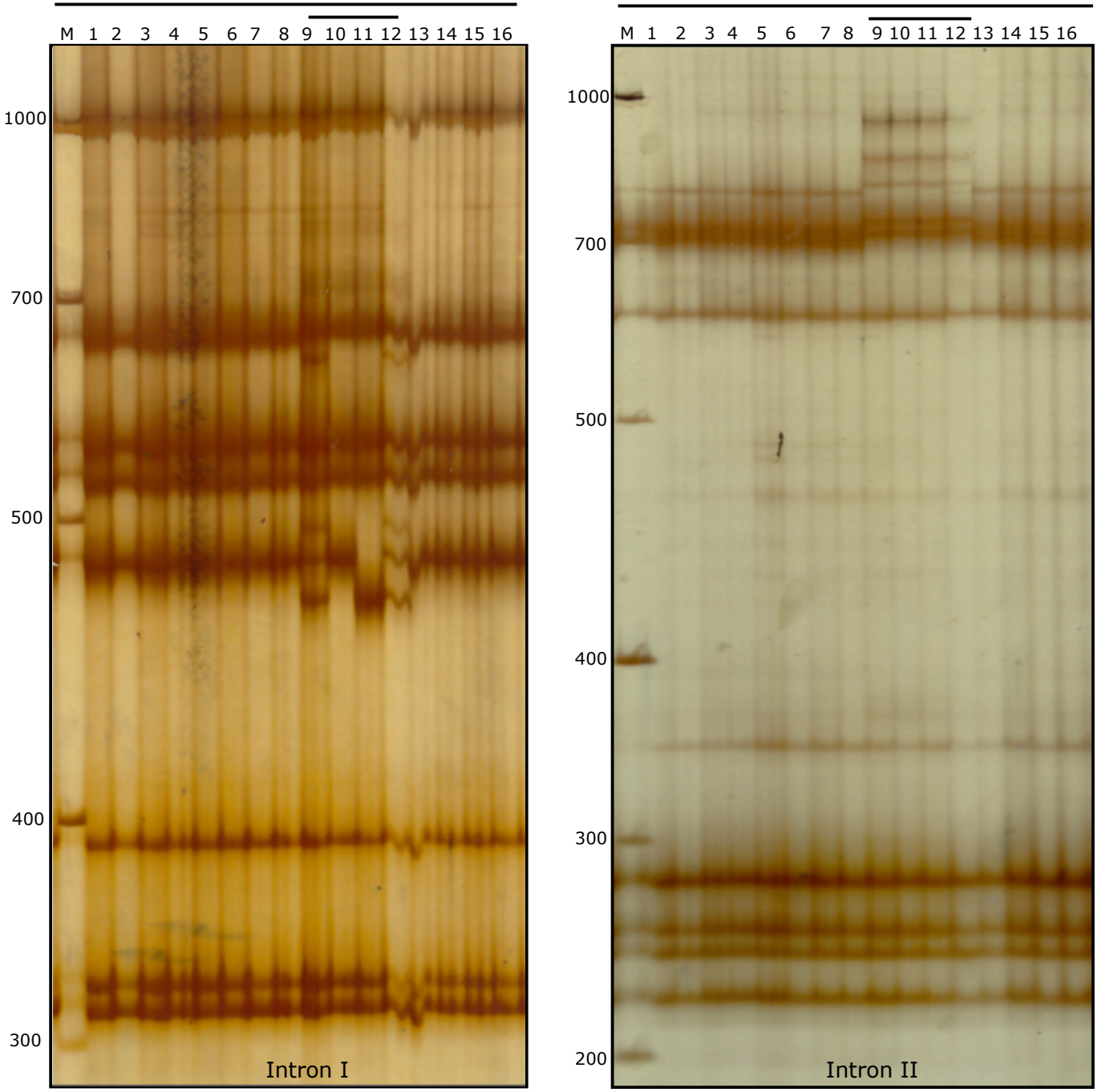

Figure SF5. Classification of *J. curcas* accessions based on seed/oil properties listed in Table S2.

Figure SF5a

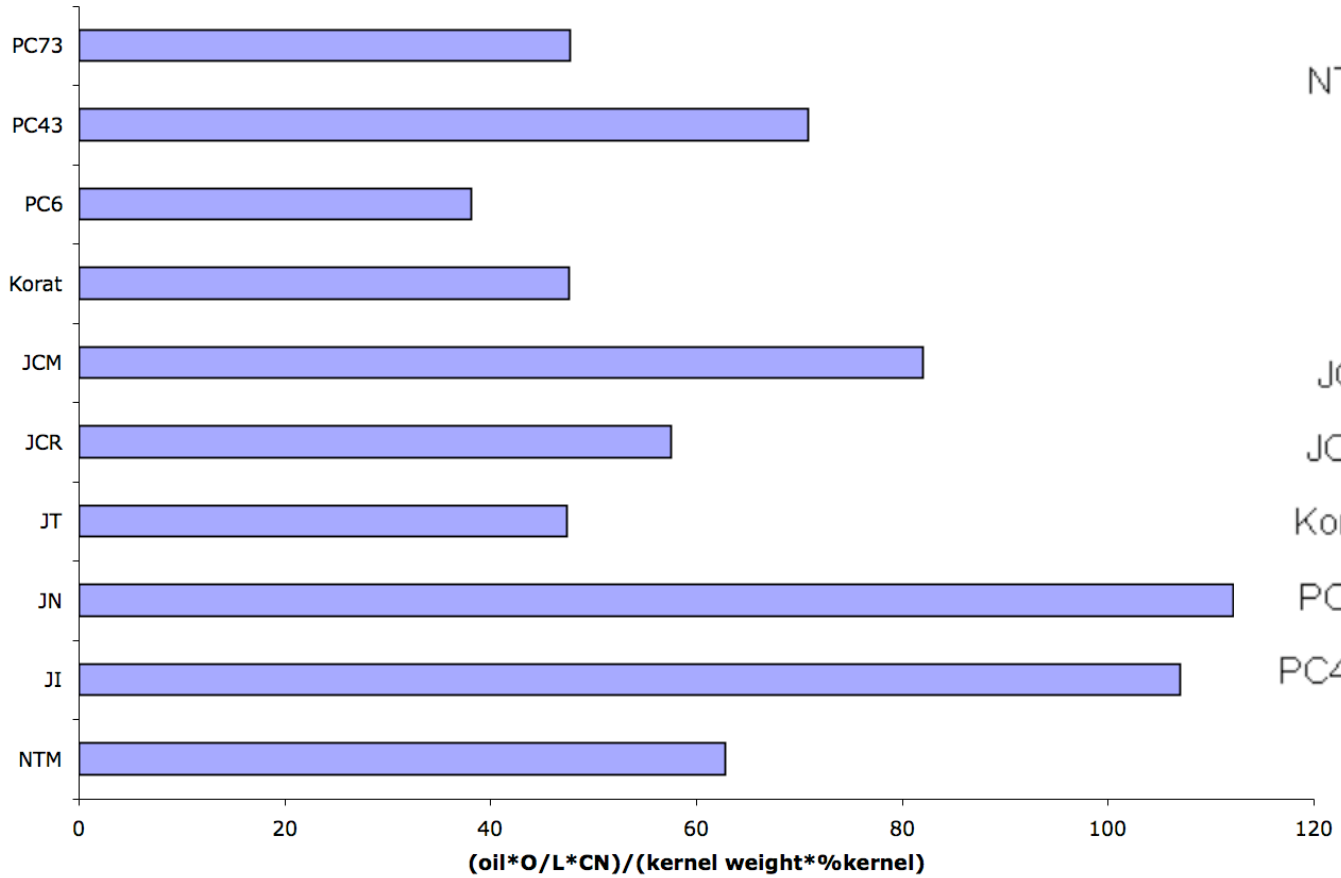

Figure SF5b

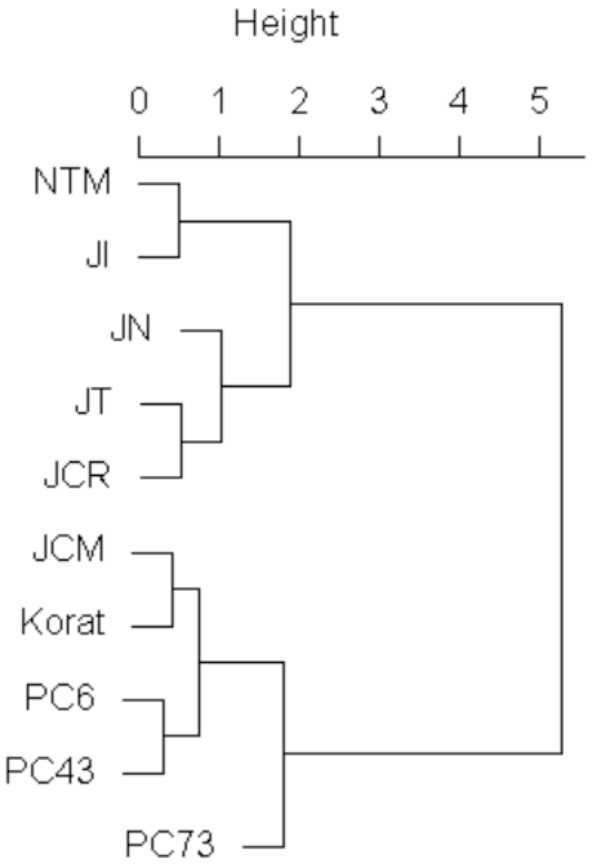

Table ST1. Global origin of *Jatropha* accessions and species used

Continent	**Country**	***J. curcas* accessions**	***J. spp* accessions**
Asia	India	10	6*
	Thailand	7	1[§]
Africa	Egypt	1	
	Mali	1	
	Nigeria	2	
	Tanzania	4	
Americas	Cape Verde	1	
	Costa Rica	4	
	Ecuador	1	
	Mexico	1	
	Paraguay	1	
	Surinam	4	
	Uganda	1	
Total		**38 +**	**7 = 45**

**J. glandulifera, J. gossypifolia, J. integerrima, J. multifida and J. podagrica, J. maheshwarii,*

[§]*J. podagrica*

Table ST2a. *Jatropha curcas* seed properties relevant to biodiesel

Name	Origin	Seed			Kernel			Shell	Kernel	Shell	Kernel
		Weight (gm)	Diameter (mm)	Length (mm)	Weight (gm)	Diameter (mm)	Length (mm)	Weight (gm)	% Weight	% Weight	Density gm/cc
NTM	Mexico	0.692	10	16.99	0.444	8.56	13.91	0.248	64.1	35.9	0.1
JI	India	0.633	11.33	17.6	0.422	9.19	13.7	0.211	66.66	33.34	0.089
JN	Nigeria	0.653	11.1	17.8	0.435	8.92	14	0.217	66.65	33.35	0.093
JT	Thai	0.682	11.45	17.5	0.454	9.29	14.33	0.227	66.67	33.33	0.089
JCR	Thai	0.82	11.46	19.23	0.519	9.68	15.24	0.301	63.3	36.7	0.088
JCM	Thai	0.745	11.58	19.31	0.434	8.84	15.27	0.311	58.28	41.72	0.089
Korat	Thai	0.843	11.53	18.98	0.529	9.52	14.66	0.313	62.83	37.17	0.096
PC6	Thai	0.918	11.59	19.19	0.537	9.25	14.55	0.381	58.45	41.55	0.1
PC43	Thai	0.844	11.3	19.63	0.532	8.92	14.79	0.312	63	37	0.098
PC73	Thai	0.877	11.25	19.24	0.511	9.07	15.25	0.365	58.31	41.69	0.1

Table ST2b. *Jatropha curcas* oil properties relevant to biodiesel

	Oil	FAMES (%)						SN	IV	CN	GE
	%(w/w)	P	S	O	L	M	O/L				kCal/mol
NTM	37.4	14.9	7.6	37.5	30.6	9.4	1.22	177.01	88.65	56.13	3054.04
JI	52.2	11.6	6.6	48.1	32.1	1.6	1.49	189.31	98.08	53.06	2826.72
JN	51.1	11.1	6.5	49.9	30.1	2.4	1.65	189.03	97.82	53.17	2831.56
JT	49.5	11.9	6.1	36.1	44.6	1.3	0.81	191.34	112.15	49.49	2790.88
JCR	37.4	15.9	10.8	40.3	30.3	2.7	1.33	188.54	89.01	55.22	2830.78
JCM	39.4	15.1	10.3	43.6	28.8	2.2	1.51	189.91	89.01	55.01	2817.04
Korat	37.8	15.5	8.2	38.8	35	2.5	1.11	189.42	93.33	55.09	2825.45
PC6	30.7	16.1	8.1	38.8	34.9	2.1	1.11	190.64	93.33	54.91	2804.55
PC43	49.6	14.6	8.1	42	32.9	2.4	1.27	189.75	93.33	55.04	2819.87
PC73	38.1	19.4	9.5	35.4	32.9	2.8	1.07	189.01	89.11	55.15	2832.52

Table ST2c. Seed oil properties of *Jatropha* species

Species	Oil	FAMES (%)						SN	IV	CN	GE
	%(w/w)	P	S	O	L	M	O/L				kCal/mol
J. integerrima	40.2	8.64	4.39	11.98	74.75	0.24	0.16	190.90	139.21	43.57	2796.12
J. gossypifolia	47.9	11.33	5.16	23.05	59.95	0.51	0.38	190.68	123.16	47.21	2801.16
J. multifida	50.1	17.86	5.32	26.32	49.15	1.35	0.53	190.14	107.33	50.85	2811.64
J. podagrica	42.7	8.92	6.13	24.47	59.43	1.05	0.41	189.20	123.48	47.36	2826.44
J. glandulifera	53.3	7.68	7.92	20.33	64.05	0.02	0.31	190.96	127.91	46.10	2796.04