Morphometric, Molecular and Biochemical characterization of *Jatropha* germplasm of North-East India

A thesis submitted to India Institute of Technology Guwahati

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Doctor of Philosophy in Energy

by

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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CERTIFICATE

I do hereby declare that the matter embodied in this thesis entitled "***Morphometric, Molecular and Biochemical characterization of Jatropha germplasm of North-East India***" is the result of research investigations carried out by me in the Centre for Energy, Indian Institute of Technology Guwahati, India.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

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CERTIFICATE

This is to certify that the work described in the thesis entitled "***Morphometric, Molecular and Biochemical characterization of Jatropha germplasm of North-East India***" submitted by Ms. Adreeja Basu (07615101) for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out at the Centre for Energy, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree. The thesis has fulfilled all requirements as per the regulations of the institute and in my opinion has reached the standard needed for submission.

November, 2013

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"Arise, awake, and stop not till the goal is reached"

- Swami Vivekananda

Dedicated to my late grandparents

Smriti & Himan Ray and Niharika & Poet Sunirmal Basu

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ABSTRACT

Physic nut (*Jatropha* curcas L.) is emerging as a sustainable alternative source of bio-fuel for mitigation of global energy crisis. However, profitable exploitation of *J. curcas* through large scale production schemes is still in its infancy mainly due to unpredictable seed yield traits and seed toxicity. Furthermore, the plant suffers from narrow genetic base. Identification of promising populations of *J. curcas* from wild habitat or distinct agroclimatic regions is of foremost importance for overcoming this constraints and development of superior varieties in breeding programs. However, in spite of increasing global importance of *J. curcas* as a bioenergy crop, till date, very little comprehensive studies on estimation of morphological and biochemical characters of *J. curcas* along with assessment of genetic variation within the germplasm has been conducted for screening and identification of elite and genetically divergent planting materials.

With an objective to identify promising *J. curcas* populations, twenty nine populations were collected systematically from distinct agro-climatic regions of North-East India. Four populations from other parts of India were also included in the study as outgroup. In the present investigation, the morphological variation of *J. curcas* populations were assessed using agronomically important morphometric traits like plant height, collar diameter, canopy spread, floral sex ratios and yield associated traits like 100 seed weight and total seed yield. Highly significant differences in phenotypic characters were observed among *J. curcas* populations. The highest broad-sense heritability (%) was recorded for M: F ratio (88.07) and the lowest for canopy spread (66.39). The morphometric characterization of *J. curcas* populations on the basis of cluster and principal component analyses depicted that association among populations is independent of geographical origin. Seven promising *J. curcas* populations (IITJC7, IITJC15, IITJC19, IITJC21, IITJC22, IITJC24 and IITJC28) identified from North-East India on the basis of morphological characterization can be used as potential starting material for *J. curcas* improvement ventures.

Random markers (RAPD and ISSR) and curcin gene-specific marker based molecular fingerprinting techniques were employed for estimation of genetic diversity of 33 *J. curcas* populations. The average percentage of polymorphism was found to be 67.71, 66.13 and 67.09 for RAPD, ISSR and combined RAPD and ISSR. However, the curcin specific markers showed monomorphism across all 33 *J. curcas* populations. For all three datasets (RAPD, ISSR, combined RAPD and ISSR) genetic diversity estimates were comparatively high for North-East populations (Nei's gene diversity, H = 0.1820, 0.1749 and 0.1716 respectively; Shannon's Information Index, I = 0.2798, 0.2700 and 0.2701 respectively) and low for outside North-East populations (H=0.0935, 0.0619 and 0.0823 respectively; I = 0.1359, 0.0891 and 0.1194 respectively). This indicated that *J. curcas* populations from North-East India still maintain relatively high level of genetic diversity. Both cluster and principal component analysis depicted that the populations from Arunachal Pradesh, Meghalaya and Tripura are genetically more distinct than other populations. It was concluded from this study that *J. curcas* populations from North-East India are genetically more diverse than populations from other parts of India. It was inferred that the populations IITJC5, IITJC6, IITJC7, IITJC12, IITJC13, IITJC14 and IITJC15 have maintained the maximum genetic diversity and therefore may contribute substantially towards broadening the genetic base of *J. curcas* germplasm.

J. curcas seeds are an attractive feedstock of biofuel. However, the toxic phorbol esters present in *J. curcas* seeds can form life risk for bioprocess workers during oil extraction. Furthermore, the presence of phorbol esters also restricts the use of left-over seedcakes as animal feed. Thus, a study was conducted to estimate the phorbol ester concentrations of twelve *J. curcas* populations, identified as promising through phenotypic and molecular characterization. The aim of this study was to identify *J. curcas* populations having comparatively low phorbol ester content. It was found that the *J. curcas* seeds from Mawhati (IITJC12) have lowest phorbol ester content (1.02 mg/g). However, the seeds from Makum (IITJC22) have maximum phorbol ester content (2.75 mg/g).The eco-geographical region wise variation of total phorbhol ester content of *J. curcas* seeds was also observed. It was revealed that *J. curcas* seeds from geographically isolated states of Meghalaya and Arunachal Pradesh

contain comparatively low concentration of phorbol esters. From this study, three *J. curcas* populations, IITJC12 (1.02 mg/g of seed kernel), IITJC7 (1.15 mg/g of seed kernel) and IITJC15 (1.38 mg/g of seed kernel) were identified as low phorbol ester containing populations. These populations can later be used for further improvement of *J. curcas*.

The genus *Jatropha* has significant economic importance. Understanding the phylogenetic relationship of *Jatropha* species is prerequisite for successful interspecific hybridization. In this study, the genetic relationship of three *Jatropha* species *J. curcas*, *J. podagrica* and *J. gossypifolia*, commonly available in North-East India, were determined using nuclear ITS region and chloroplast *matK* gene as markers. The size of the nrDNA ITS region in *Jatropha* species ranged from 790 to 793 bp. No size variation was obtained among cpDNA *matK* regions. The ITS regions of *Jatropha* species showed 111 variable sites. On the other hand, 4 variable sites were detected in *matK* region. The ITS and *matK* sequence based Maximum parsimony Trees revealed maximum interspecific distance between *J. curcas* and *J. podagrica*. For *Jatropha*, nrDNA ITS seemed to be better marker than cpDNA *matK*. The indels and substitutions detected in this study can later be exploited efficaciously for identification of *Jatropha* species. Furthermore, the results obtained in this study will later provide valid guidelines for collection, conservation and characterization of *Jatropha* genetic resources.

The comprehensive study involving morphometric, molecular and biochemical traits elucidated that *J. curcas* populations from North-East India are morphometrically and biochemically more elite and genetically more diverse than the populations from other parts of India. It can be concluded that programs of systemic and selective breeding of *J. curcas* can be carried out with the aim of producing promising and genetically distinct varieties with low seed phorbol ester content using the elite populations identified in this study as potential starting material. The ITS and *matK* specific sequence data generated in this study can later be used for development of *Jatropha* species specific SNPs.

ABBREVIATIONS

100SW	100 seed weight
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ANFs	Anti-nutritional factors
ANOVA	Analysis of variance
CD	Collar diameter
cDNA	complementary DNA
CI engine	Compression-ignition engine
cpDNA	chloroplast DNA
CPTs	Candidate Plus Trees
CS	Canopy spread
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of variation
DGAT	Diacylglycerol acyltransferases
DNA	Deoxyribonucleic acid
EST	Expressed sequence tags
G3P	glycerol-3- phosphate
GPAT	Glycerol-3-phosphate acyltransferase
GWAS	Genome-wide association study
H	Nei’s gene diversity
h^2_{bs}	Broad-sense heritability

I	Shannon’s Information index
ISSR	Inter Simple Sequence Repeats
ITS	Internal transcribed spacer
LPAAT	Lysophosphatidic acid acyltransferase
M:F	Male to female flower ratio
MAB	Marker assisted breeding
MAS	Marker Assisted Selection
matK	maturase kinase
MEGA	Molecular evolutionary genetics analysis
MH	Maleic hydrazide
MP	Maximum Parsimony
MSAP	Methylation sensitive amplification polymorphism
MUFA	Monounsaturated fatty acid
Na	Observed number of alleles
NBPGR	National Bureau of Plant Genetic Resources
NCBI	National Center for Biotechnology Information
Ne	Effective number of alleles
NOVOD	National Oilseeds and Vegetable Oils Development
NPR	Non-expressor pathogen related protein
nrDNA	nuclear DNA
PB	Paclobutrazol
PCA	Principal components analysis
PCR	Polymerase chain reaction

PDAT	Phospholipid:diacylglycerol acyltransferase
PGRs	Plant growth regulators
PH	Plant height
PMA	Phorbol 12-myristate-13-acetate
PUFA	Polyunsaturated fatty acid
QTLs	Quantitative trait loci
R	Transition/Transversion bias
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RIP	Ribosome inactivating protein
RNA	Ribonucleic acid
RNAi	RNA interference
SNP	Single nucleotide polymorphism
SPR	Subtree Pruning Regrafting
SSR	Simple sequence repeats
STMS	Sequence tagged microsatellites
TAG	Triacylglycerol
TBE	Tris-borate-EDTA
Ti	Transition
TILLING	Targeting Induced Local Lesions In Genome
TRV	Tobacco Rattle Virus
TSY	Total seed yield
Tv	Transversion

UPGMA	Unweighted pair group method with arithmetic mean
V_G	Genotypic variance
VIGS	Virus-induced gene silencing
V_P	Phenotypic variance

UNITS

ha	hectare
m	meter
mm	millimeter
Kg	Kilogram
ha	hectare
m	meter
mm	millimeter
^{o} C	degree Celsius
MJ/Kg	mega joule per kilogram
ng	nanogram
µl	microliter
µM	micromolar
min	minute
bp	base pair
mg/g	milligram per gram
g	gram
ml	milliliter
rpm	revolution per minute
µg	microgram
Å	angstrom
nm	nanometer

U	unit
V	volt
kb	kilobase
cm	centimeter
Mb	megabase

Chapter 1

Introduction

1.1 Introduction

The rapid increment of world energy demand, significant diminution of fossil fuel (oil, gas and coal) with escalation of petroleum prices and the growing perils of global warming has prompted alternative energy sources to become an inevitable substitute for recovery of industrial and economical development. Fuels derived from biological origins possessing almost same heat values as that of petro-diesel have given excellent and environment friendly opportunities to generate sustainable energy. The concept of using biodiesel in diesel engine was first introduced in 1885 by Dr. Rudolf Diesel who used peanut oil as fuel (Murugesan et al. 2009). Rudolf Diesel, in 1911, was quoted as saying "the use of vegetable oils for engine fuels may appear insignificant today. But such oils may gain the importance in future as petroleum and the coal tar products" (Babu and Devaradjane 2003).

However, the high price of vegetable oils such as sunflower oil, soybean oil and palm oil will lead to the increase of the overall production cost of the biodiesel as well. Hence, it is better to use the non-edible oils for biodiesel production. Thus, the exploitation of non-edible oil seeds viz. *Jatropha curcas*, *Pongamia pinnata*, *Ricinus communis* etc. as feedstock for biodiesel will be economical in comparison to vegetable oil-based biodiesel.

The fact that the oil obtained from *Jatropha curcas* seeds upon transesterification can provide better quality biodiesel with high cetane number when compared with other oil-seed plants has created a worldwide surge of interest in this plant. Furthermore, hardiness, rapid growth, short gestation period, easy propagation, drought endurance together with wide adaptability to adverse agro-climatic conditions makes this plant ideal for wasteland reclamation (Jones and Miller 1991). Large scale cultivation remains the single most important determinant that will finally prognosticate the success of *J. curcas* as a bio-fuel crop. However, the limited information about the genetics of this species, narrow diversity in germplasm, inconsistent seed yield, varying seed oil content, high male to female flower ratio and susceptibility to insects and diseases pose major constraints in successful utilization of *J. curcas* as a bio-fuel crop (Reddy and Sudheer 2010).

The seeds of *Jatropha* are genetically heterozygous as it forms artificial and natural hybrid complexes readily (Prabakaran and Sujatha 1999). Because of heterozygous nature, the seed oil content varies widely (4-40%) within the species (Jha et al. 2007). Furthermore, adaptation of *Jatropha* to a wide range of edaphic and ecological conditions along with its semi-wild distribution at diverse geographical locations has harbored a considerable amount of genetic diversity to be exploited for potential realization (Rao et al. 2008). In India, proper pedigree and provenance records of *Jatropha* for identification of elite accessions for region specific cultivation has not been adequately investigated up till now with systematic documentation (Ranade et al. 2008).

Morphological trait based characterization of *Jatropha* from different agro-climatic regions is definitely prelusive for identification of agronomically promising genotypes with respect to high female to male flower ratio, high seed yield potential with high oil content, low gestation period and reduced plant height. For identification of high yielding genotypes, determination of magnitude of variation present in the available germplasm, interrelationship of plant growth pattern with seed yield traits, extent of environmental influence on these factors, heritability and genetic gain of the plant material is of utmost importance. A few reports on morphological traits have indicated existence of considerable morphometric divergence among the accessions in *Jatropha* (Rao et al 2008; Ginwal et al. 2004; Saikia et al. 2009; Srivastava et al. 2011; Shabanimofrad et al. 2013). However, till date, comprehensive studies on vegetative and yield attribute for systematic identification of elite plants is distinctly lacking in *J. curcas*.

In addition to phenotypic characterization, molecular markers based characterization allows the detection of DNA polymorphism that can be efficiently used for selection of highly divergent varieties as well as marker-assisted elimination of redundancy in *Jatropha* germplasm. Besides, molecular markers are more reliable for precise characterization of the germplasm as they are not influenced by variable environmental conditions or plant phenology. In recent years, PCR-based molecular-typing approaches like Random Amplified Polymorphic DNA (RAPD) (Williams et al. 1990) and Inter Simple Sequence Repeats (ISSR) (Zietkiewicz et al.

1994) have been efficiently employed to analyze inter- and intra-specific genetic diversity in *Jatropha* (Basha and Sujatha 2007; Pamidimarri et al. 2009b; Pamidimarri et al. 2010; Gupta et al. 2008).

The proteinaceous residues obtained after extraction of oil from *J. curcas* seeds have high nutrient value. However, the phytochemical analysis of *J. curcas* seeds revealed the presence of high concentration of co-carcinogenic phorbol esters in the endosperm (Adolf et al. 1984, Goel et al. 2007). Phorbol ester, the main toxic agent responsible for *J. curcas* toxicity, limits the utilization of the protein rich residues as animal feed. Phytochemical characteristic based screening of *J. curcas* germplasm for identification of low phorbol ester containing varieties will help in the widespread acceptance of *Jatropha* seedcake as animal feed.

Thus, the comprehensive study of phenotypic, genetic and phytochemical traits of *J. curcas* has become highly imperative for attaining systematic collection and effective conservation of diverse germplasm. The agro-morphological and phytochemical markers employed along with molecular markers in characterization of *J. curcas* germplasm will lead to the identification of genetically diverse elite plants possessing superior seed yield traits and low phorbol ester content. A careful perusal of literatures reveal that except a single preliminary attempt to correlate morphometric and molecular diversity (Sunil et al. 2011), comprehensive work on collection of wild varieties of *J. curcas* germplasm and complementation of their morphometric, molecular and biochemical traits is still in its infancy.

North-East India, being a part of both trans-Himalayan mountainous zone and Indo-Burma biodiversity hotspot, is the richest plant diversity reservoir of India (Myers et al. 2000; Mao et al. 2009). Encompassing an area of 2,62,379 sq. km., North-East India has been broadly divided into two major biogeographic zones – Eastern Himalaya (Arunachal Pradesh and Sikkim) and North-East India (Assam, Nagaland, Manipur, Meghalaya, Sikkim and Tripura) based on floristic composition and climatic conditions (Rodgers and Panwar 1988). *J. curcas* has now become well-naturalized plant species in this unique biogeographic region (Mazumdar et al. 2012). Till now, little research has been conducted on morphometric, molecular and biochemical characterization of the plant for utilization of the untapped genetic resources of *J.*

curcas germplasm from North-East India. In a preliminary attempt to study the morpho-physiological variation and growth performance of *J. curcas* germplasm, Saikia et al. (2009) reported satisfactory growth performance of accessions from North-East India. Kumar et al. (2013) recently has reported the genetic diversity analysis of *J. curcas* germplasm covering only two states (Assam and Meghalaya) of North-East India. However, major part of this floristically enriched region has not been thoroughly studied hitherto. Furthermore, there are no reports on comprehensive and correlative studies for screening and identification of promising *Jatropha* accessions from North-East India. This has necessitated carrying out coherent work on finding the phenotypic, genotypic and biochemical variations of *J. curcas* germplasm of North-East India and select elite accessions among planted or naturalized population of *J. curcas*.

The genus *Jatropha* is morphologically diverse encompassing many potential species with considerable economic and medicinal attributes. However, very little is known about the genetic relatedness and phylogenetics of the *Jatropha* species. Determination of genetic relationships among species is critical for the management of genetic resources and success of interspecific hybridization. DNA fingerprinting techniques involving organelle specific markers have been widely accepted as powerful fingerprinting tools for finding phylogenetic relationship between different species. Molecular phylogenetic studies involving nuclear DNA ITS along with chloroplast DNA *matK* markers will significantly increase our understanding of the systematic of *Jatropha*. This will further facilitate in establishment of molecular database for breeding purpose.

In the present study, a comprehensive effort has been made to correlate morphological variation, genetic diversity and biochemical analysis of *J. curcas* germplasm of North-East India which will enable identification of diverse provenances with promising traits. The study also aims to elucidate the extent of genetic variability of *J. curcas* populations from North-East with the populations from other parts of India. Organelle specific marker based fingerprinting is adapted to estimate genetic distance between *J. curcas* and two of its related species, *J. gossypifolia* and *J. podagrica* for establishment of molecular database useful in breeding programs.

1.2 Objectives

The present investigation is thus carried out with the broad objectives to analyze the extent of phenotypic, molecular and biochemical variations in North-East genotypes of *Jatropha* when a comparative study is undertaken with the genotypes from other parts of India. The objectives are outlined as:

- Systematic collection and morphometric analysis of *J. curcas* populations
- Molecular marker based genetic diversity studies of *J. curcas*
- Phytochemical characterization of *J. curcas* for low phorbol ester content
- Studies on genetic relationship of *J. curcas* with *J. podagrica* and *J. gossypifolia*

1.3 Organization of Thesis

This thesis is organized into eight chapters. The literature review on assessment of phenotypic, genetic and biochemical diversities of *Jatropha* germplasm and approaches for varietal improvement of *J. curcas* are addressed in Chapter 2. The study related to morphological characterization of *J. curcas* germplasm of North-East India has been presented in Chapter 3. Chapter 4 elaborates the genetic diversity studies of *J. curcas* germplasm of North-East India employing random and gene-specific markers. The variation in total phorbol ester content of *J. curcas* populations have been represented in Chapter 5. Chapter 6 elucidates the interspecific relationship of *Jatropha* using nuclear DNA and chloroplast DNA specific markers. In chapter 7, finally the conclusions drawn from these studies have been discussed. The scope of future work has also been highlighted. In chapter 8, the list references cited in this thesis work has been provided.

Chapter 2

Literature Review

2.1 Biodiesel: A sustainable fuel

The sharp rise in the price of petroleum, gradual depletion of non-renewable fossil fuel reserves and increasing concern regarding global warming has incremented the need for an alternative, renewable, economically viable and environmentally friendly energy resource. The exploitation of biofuels such as biodiesel (produced by transesterification of vegetable oils or animal fats in presence of methanol) and bioalcohol (produced by fermentation of sugar and starch derived from sugar and starch crops, or lignocellulosic biomass) is a viable alternative for mitigating the rising global energy crisis. Biofuel offers unique opportunities for generation of environment friendly sustainable energy along with following distinct advantages: (1) easy availability and renewability, (2) formidably low emission values for Green House Gases that have increasingly destructive effect on the environment (3) high rate of biodegrability and lastly, (4) good lubricant properties which substantially contributes towards a superior wear performance of the fuel (Agrawal 2007).

In upcoming years, the increase in demand for biofuel will also subsequently trigger the demand of feedstock. A list of potential feedstock for production of biofuel has been consolidated in Fig. 2.1. Currently, edible resources (rapeseed, sunflower, palm, soybean, sugarcane, corn and others) are mainly used as feedstock for the production of “first-generation” biofuel. However, the incessant and large-scale production of biofuel from edible resources is not feasible. Oil from edible resources only meets 5-6% of world’s energy demand. If all the available edible oil resources are used for bio-fuel production, this will pose a threat in the supply of food oil. This limitation has prompted the advent of “second-generation” biofuel from non-edible resources. Lignocellulosic biomass from perennial grasses (switchgrass, and miscanthas) and trees (poplar and willow) can serve as dedicated feedstock for production of biofuel. However, the presence of high concentrations of lignin and complex polysaccharides in lignocellulosic biomass makes the production of fermentable sugars difficult and as a consequence the ethanol obtained as a final product may become economically prohibitive (Gao et al. 2012).

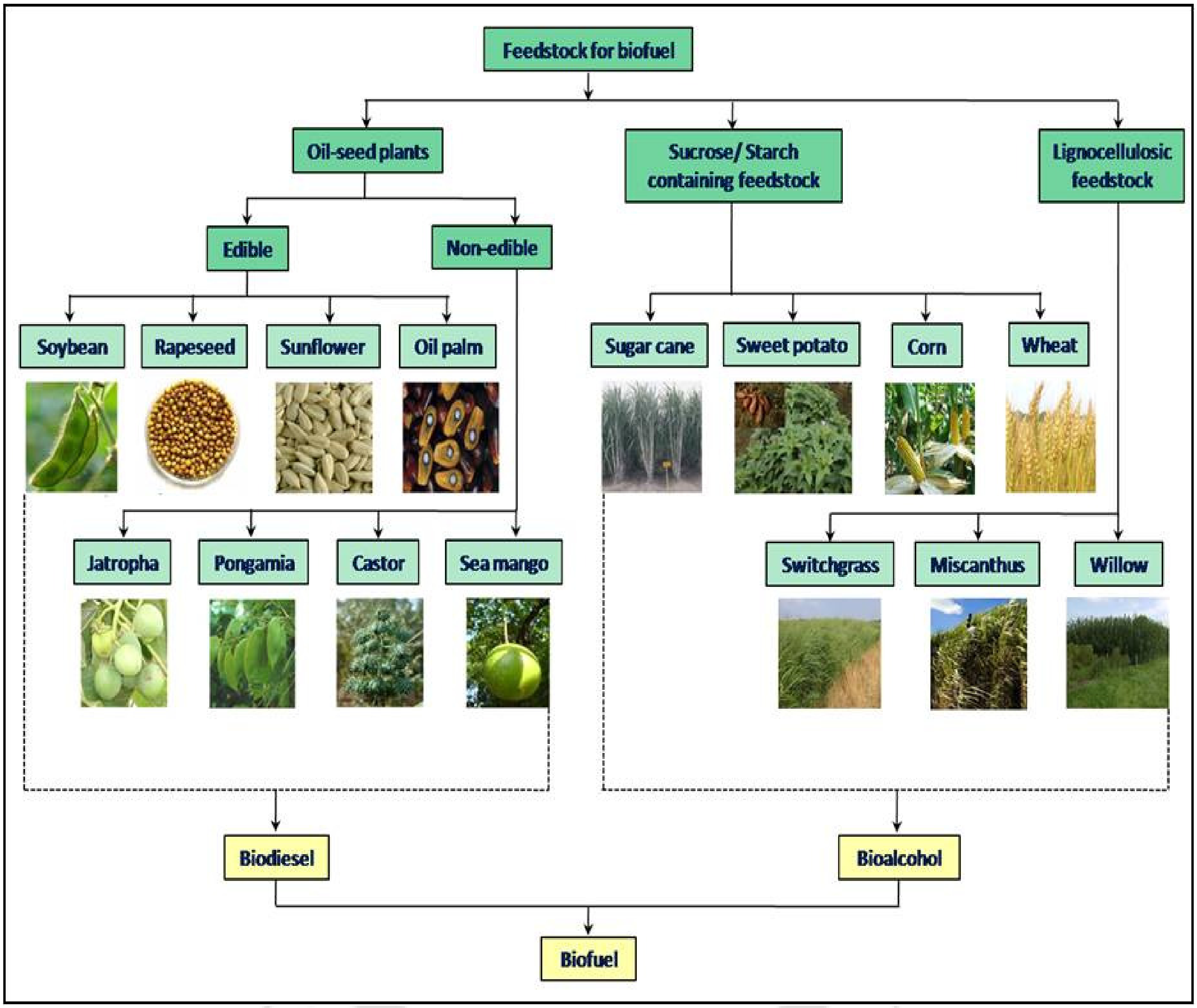


Fig. 2.1 Feedstocks for biofuel (Atabani et al. 2012; Kumar and Sharma 2011)

Non-edible oil seeds obtained from oleaginous plants like *Jatropha curcas* (ratanjyot), *Ricinus communis* (castor), *Pongamia pinnata* (karanj), *Madhuca indica* (mahua), *Hevea brasiliensis* (rubber tree) may serve as potential feedstock for production of biodiesel in tropical and sub-tropical climates across the developing countries (Gui et al. 2008; Balat 2011). *Jatropha*, in particular, has extra advantages over other perennial non-edible oil seed plants. Being drought tolerant, it is capable of surviving in abandoned and fallowed agricultural land. It is highly disease resistant and not eaten by livestock (Becker and Makkar 2008). However, the most important advantage offered by *Jatropha* in comparison to other oilseed crops like castor and cotton is, the co-products produced during *Jatropha* seed oil extraction can be successfully employed for production of high concentration of bioethanol (Visser et al. 2011).

2.2 *Jatropha curcas*: a potential biofuel crop

Jatropha, the undemanding oil plant, is considered as one of the most promising potential oil source to produce biodiesel in India. There are several advantages associated with *Jatropha* (Kumar and Sharma 2008; Achten et al. 2008):

- *J. curcas* is a shrub. Therefore, it is easier to harvest than large trees. Moreover, in comparison with other bio-diesel plants (for e.g. *P. pinnata*), it has much shorter gestation period. Once this plant becomes an adult, it can continue producing seeds for 50 years.
- *Jatropha* is drought resistant. It is also adapted to various soil conditions, including low-nutrient soils such as sandy, saline and stony soils. Furthermore, it requires less nutrients to survive and therefore can be grown on wasteland which reduces land competition with food crops. In addition, it can serve as a good plant material for eco restoration, carbon restoration and sequestration in all types of wasteland.
- *Jatropha* can propagate easily from plant cuttings or seeds and grows relatively quickly.
- The plant is highly pest and disease resistant and not consumed by the cattle.

- *J. curcas* plant has diverse uses and medicinal properties. The seed oil, besides being an important feedstock for biodiesel, was found to exhibit insecticidal, molluscidal, fungicidal and nematicidal properties. The nitrogen rich seed cake left after extraction of oil is excellent organic manure while the seed husk is an excellent feedstock for gasification (Vyas and Singh 2007).

2.3 Botanical description

The genus *Jatropha* belongs to tribe *Joannesieae* of the subfamily *Crotonoideae* in the *Euphorbiaceae* family. *Jatropha* is morphologically diverse containing approximately 170 known old and new world woody species. *J. curcas* (Physic nut) is a perennial deciduous shrub to small evergreen tree with a height of three to five meters. *J. curcas* shows articulated growth, with a morphological discontinuity at each internode, and its dormancy is induced by rainfall and temperature variations. Flowering in *J. curcas* occurs during the wet season and two flowering peaks are often seen, i.e. during summer and autumn. The plant is monoecious and flowers are unisexual with occasional occurrence of hermaphrodite flowers (Dehgan and Webster 1979). The flowering season, flowering flushes and floral sex ratio of *Jatropha* depend largely upon temperature, moisture level and fertility of soil (Kant and Wu 2011). The flowers are entemophilous. Each inflorescence yields a bunch of approximately 10 or more ovoid trilobed fruits with seeds inside (Singh 1970). The fruits mature 2-4 months after flowering. The mature *J. curcas* seeds contain about 30–50% oil with a high percentage of monounsaturated oleic and polyunsaturated linoleic acid which is an ideal feedstock for producing biodiesel (Pramanik 2003). *J. curcas* seeds also contain a wide range of phytochemials some of which are toxic to humans and animals. Phorbol esters, a group of tetracyclic diterpenoids, have been identified as the main agent responsible for *J. curcas* toxicity (Makkar et al. 1997).

2.4 Origin and distribution

J. curcas is a native of tropical America, with the original region of distribution in Mexico and Central America (Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama). From the Caribbean, where this species had been already used by the Mayas in the 16th century, *J. curcas* was probably distributed by Portuguese seafarers via the Cape Verde Islands and Guinea Bissau to other countries in Africa and Asia (India, China and Indonesia) (Schmook and Seralta-Peraza 1997; Heller 1997). Nowadays, it grows pantropically, from Brazil to the tropical islands of Fiji. *J. curcas* have been well naturalized in different eco-geographical conditions of India. It has widely spread in wild and semi-wild status as hedge plant, as fence on field boundary, road-side and forest-ecosystems in various states of India (Sunil et al. 2012a).

2.5 Agronomic requirements

Jatropha can thrive well in hot conditions. It can even survive in lower temperatures and withstand a light frost. It can thrive under a wide range of rainfall ranging from 250 to 3000 mm per annum (Foidl et al. 1996). Its water requirement is extremely low. In arid and semi-arid areas and in prolonged rainless periods, the plant sheds its leaves to reduce transpiration loss. It grows on well-drained soils with good aeration; however it is also well adapted to marginal soils with low nutrient content as well as moderately sodic and saline soil (Banerji et al. 1985). Furthermore, *J. curcas* is suitable for ecorestoration and mitigating soil erosion. *J. curcas* has spread beyond its original distribution because of its hardiness and adaptability to wide range of agro-climatic conditions.

2.6 Diversity assessment in *Jatropha* germplasm

The progress of *Jatropha* improvement programs largely relies on the exploitation of genetic variation for a desired morphometric or biochemical trait of agronomic significance within the germplasm repertoire. The very fact that *Jatropha* has adapted itself to a wide range of environmental conditions suggests that it has possibly harbored a significant amount of genetic variation within the germplasm which can be exploited for potential realization (Rao et al. 2008). During germplasm exploration survey, comprehensive characterization of morphometric, genetic and phytochemical character of *Jatropha* germplasm is imperative for identification of unique accessions having considerable genetic variability and desired agronomic traits (Fig. 2.2). Furthermore, germplasm characterization will assist in broadening *Jatropha* genetic resource through introduction of diverse accessions. Studies on morphometric, molecular and phytochemical analyses of *Jatropha* germplasm have been carried out randomly throughout the world. In this section, an attempt has been made to systematically compile the majority of *Jatropha* diversity studies carried out to date.

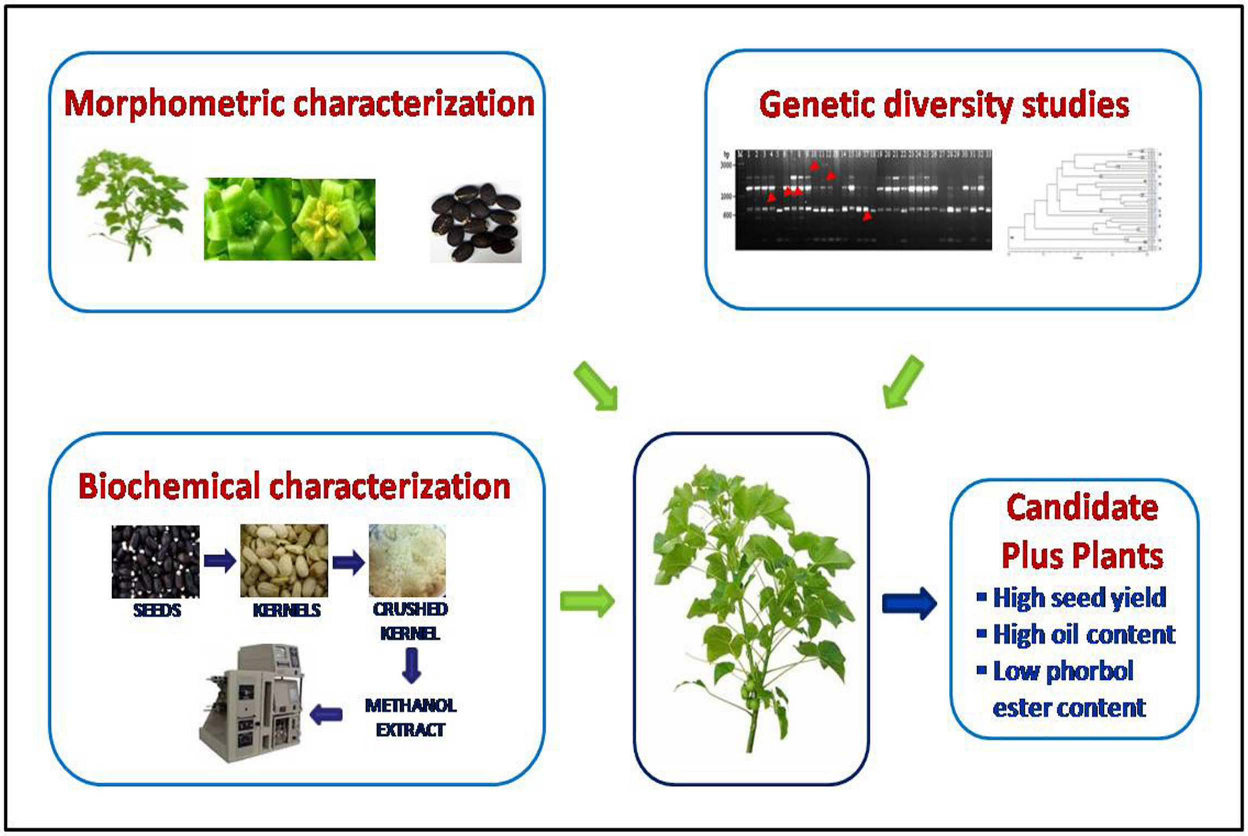


Fig. 2.2 Morphometric, molecular and phytochemical diversity based analyses for identification of elite *J. curcas* varieties

2.6.1 Current status of morphometric diversity studies in *Jatropha*

It is a well known fact that the success of breeding-mediated plant improvement endeavors depends largely on morphological variations of agronomically significant traits within the germplasm and determination of genetic factors responsible for the trait variation. Thus, morphometric diversity analysis of *J. curcas* germplasm is of utmost significance for a thorough investigation of extent of phenetic diversity in germplasm and identification of elite accessions for specific breeding purposes. A number of quantitative and qualitative traits such as seed yield, oil content, female to male flower ratio, increased branching, early flowering and synchronous maturity are considered as focal characters for commercial utilization of *J. curcas*. Estimation of the amount of variability present in these traits within the germplasm is essential for *J. curcas* improvement program. With the objective of assessment of agronomic trait based variation among *J. curcas* accessions, few independent morphometric diversity studies have been performed throughout the world.

2.6.1.1 Worldwide diversity of *Jatropha* germplasm

In an initial attempt to characterize the phenotypic diversity among *J. curcas* populations of Ethiopia, Hailu (2010) assessed the differences in 12 different phenotypic traits. Significant variation among the populations was observed for leaf width, average leaf area, leaf dry matter, length of leaf from width to base and apex. In a subsequent study, Santoso (2011) reported substantial variation among seed traits and seedling characteristics of West Nusa Tenggara populations of *J. curcas* from Indonesia. Unanaonwi and Nwabueze (2012) carried out an investigation focusing on early growth morphology of *J. curcas* populations from four ecological zones of Nigeria. The study revealed differences in morphometric parameters among the populations. The first attempt to screen candidate plus *J. curcas* plants in China on the basis of morphological variations and oil-yield traits resulted in identification of 6 elite plants among 80 *J. curcas* accessions from South China (Yang et al. 2010a). In an attempt to correlate phenotypic, genotypic and genetic divergence among 48 Malaysian accessions using morphological markers, Shabanimofrad et al. (2013) reported high values of genotypic coefficient of variation for total number of seeds per plant, total number of branches per plant, number of primary branches per plant and seed yields per ha along with identification of 7 elite accessions. Hitherto, only sporadic attempts have been made in different countries to assess the extent of genetic variability within local *J. curcas* germplasm using morphological traits. More systematic characterization of global germplasm involving morphological traits is required for a better understanding of the true picture of *Jatropha* morphometric diversity worldwide.

2.6.1.2 *Jatropha* diversity in India

In India, initial work on characterization and evaluation of genotypes for growth, morphology, seed characteristics and yield traits has been initiated by several independent groups with locally available germplasm. A few studies have already reported a modest level of phenotypic diversity within the *Jatropha curcas* germplasm in India. In two concurrent studies, Ginwal et al. (2004; 2005) analyzed the genetic variability associated with growth performance, oil yield and seed traits of *J. curcas* accessions collected from the Madhya Pradesh and Maharashtra. They reported the existence of a considerable amount of variability within *J. curcas* germplasm with respect to seed traits. Kaushik et al. (2007) investigated genetic variance for seed traits and oil content among 24 *J. curcas* accessions from diverse agroclimatic regions of Haryana using non-hierarchical Euclidian cluster analysis. They proposed that crossing between accessions having high inter-cluster distance may result in generation of broad spectrum of variability in successive generations. In another study, Rao et al. (2008) observed four clusters with phylogeographic patterns of genetic diversity among 32 high yielding elite trees of *J. curcas* from Andhra Pradesh for seed traits and suggested selection of trees as parents from clusters with high inter-cluster distance for development of novel hybrids. Das et al. (2008) analyzed genetic divergence among 18 provenances of *J. curcas* from Orissa, Gujarat and Rajasthan with respect to their growth performance following numerical taxonomic approach and proposed reforestation of wastelands of Orissa with the identified elite accessions. Sunil et al. (2008) documented the morphometric traits and analyzed the oil content of diverse germplasm from Andhra Pradesh and Chhattisgarh with successive identification of promising accessions. In a subsequent study, the same research group used the rarefaction method of DIVA-GIS based software for identification of potential areas having diverse plant materials with a special focus on accessions having high seed oil content, from south east coastal zone of India (Sunil et al. 2009). Gohil and Pandya (2009) evaluated extent of variability of nine *Jatropha* genotypes from Rajasthan and Gujarat based on morphometric characters. They suggested that hybridization among the genotypes of divergent clusters may facilitate improvement of *J. curcas*. Mohapatra and Panda (2010) and Pandey et al. (2010) did similar studies on morphometric and seed trait based characterization of *J. curcas* germplasm from Orissa and Madhya Pradesh, respectively, and reported independently significant morphological variations among the accessions. In an attempt to describe the variation in seed and seedling characters of *J. curcas* collected from six diverse climatic zones of India, Ghosh and Singh (2011) observed maximum diversity among provenances from sub-humid to humid eastern, south eastern uplands, semi arid lava plateaus and central highlands. Gairola et al. (2011) reported moderate variations in seed characters of *J. curcas* accessions from Uttarakhand Himalaya. Naresh et al. (2012) observed significant variations among accessions from Andhra Pradesh and Rajasthan in terms of seed characters and oil content and selected four superior accessions for further research.

Thus, most of the studies reported significant divergence among *Jatropha* accessions in terms of morphometric parameters. However, with the advent of DNA fingerprinting technology, the molecular markers have largely replaced morphometric markers in most of the *Jatropha* diversity studies.

2.6.2 Current status of molecular diversity studies in *Jatropha*

The commercial deployment of *J. curcas* as a feedstock for bio-diesel is only feasible with the development of novel varieties with a high seed yield and a high oil content that are adapted well to diverse eco-geographic conditions. However, *J. curcas* has a heterozygous genome, so improvement of the agronomic traits through traditional breeding strategies might not be efficacious (Wen et al. 2010). In recent years, molecular markers have displayed great efficacy for plant breeding in enhancing the efficiency of selection of desirable traits via marker-assisted breeding and understanding the genetic relationships, evolutionary trends and fingerprinting of genotypes. Therefore, a molecular-marker based breeding approach can become an efficient tool for genetic improvement of *J. curcas*. The study and systematic evaluation of genetic diversity within *J. curcas* germplasm with subsequent identification of unique individuals having high seed yield traits and oil content is prerequisite for marker assisted breeding (MAB). Few DNA profiling studies across the globe have already employed PCR-based markers such as RAPD (random amplified polymorphic DNA), ISSR (inter simple sequence repeats), SSR (simple sequence repeats), EST-SSRs and AFLP (amplified fragment length polymorphism) for evaluation of genetic diversity in *J. curcas* germplasm. *Jatropha* molecular biology studies carried out worldwide, so far, are confined to genetic diversity estimation of local germplasm of *J. curcas*. On contrary, independent research groups in India have carried out genetic diversity estimation at both the inter- and intra-specific level. With an objective to evaluate the current status of *Jatropha* genetic diversity, the molecular diversity studies in *Jatropha* carried up till now has been collaborated in a systematic way.

2.6.2.1 Worldwide diversity of *Jatropha* germplasm

Evaluation of global genetic diversity of *J. curcas* germplasm was initiated by Montes et al. (2008). In this study, it was observed that, among 225 accessions collected from 30 different countries, African and Indian accessions have low genetic variation while Guatemalan and other Latin American accessions have high genetic variance. Assessment of the genetic relationship between 58 *J. curcas* accessions from South China based on SSR and amplified AFLP analyses revealed narrow genetic diversity (Sun et al. 2008). In contrary to the above study, existence of moderately high level of genetic diversity was found among Chinese accessions based on ISSR analysis with respect to polymorphism rate, Shannon's information index and Nei's gene diversity (Cai et al. 2010). Shen et al. (2010; 2012) in two concurrent studies assessed the *Jatropha* genetic diversity amongst populations from China and Vietnam. It was inferred that in order to avoid inbreeding depression, it is necessary to include wild germplasm for broadening the genetic base for tree improvement program. In a similar investigation, Grativol et al. (2011) reported low genetic diversity across the *J. curcas* accessions from Brazil. In contrast to above studies, high molecular genetic diversity among *J. curcas* populations from Mexico has been reported by Pecina-Quintero et al (2011); the analysis of genetic relationships, analysis of molecular variance (AMOVA) and the diversity index all confirm a broad gene pool of the species from Chiapas, Mexico. Thus, the studies on assessment of *Jatropha* diversity across the globe confirmed the fact that for broadening the diversity in *J. curcas* genetic resources, inclusion of wild genotypes from natural habitats is of utmost importance.

2.6.2.2 *Jatropha* diversity in India

Inter-specific diversity based studies

Estimation of molecular diversity between *Jatropha* species can help in inter-specific hybridization and serve as a panacea for all the problems related with tree improvement programs of *J. curcas*. Few studies in India have reported high amount of genetic diversity within *Jatropha* species.

Multilocus marker based molecular studies

Study on phylogenetic significance of inter-specific hybridization in *Jatropha* by Dehgan (1979) was the earliest investigation on assessment of interspecific diversity in this plant. From the investigation, It was inferred that the species *J. macrorhiza, J. capensis*, *J. cathartica*, *J. multifida*, *J. podagrica*, *J. cordata*, and *J. cinerea* can be crossed unilaterally with *J. curcas*. In an attempt to investigate genetic distance between 8 *Jatropha* species based on ISSR markers, Kumar et al. (2008) reported that all the *Jatropha* species have the greatest phylogenic distance from *J. curcas*. Similar studies done by Ram et al. (2007) and Vijayanand et al. (2009) based on RAPD and ISSR markers, respectively, confirmed the molecular distinctness of *J. curcas* with respect to other species. Therefore, it is worthwhile to exploit the sister species for widening the narrow genetic base of *J. curcas* as the plant is readily crossable with most of the species when used as female parent (Ram et al. 2007). High diversity was detected in the genus *Jatropha* when organelle-specific markers in combination with nuclear markers were used to unravel the genetic relationship between eight agronomically important species (Basha and Sujatha 2009). Thus, all the studies affirmed the fact that that the molecular diversity available in the genus *Jatropha* can be potentially exploited for the improvement of *J. curcas* traits by interspecific breeding.

Organelle specific marker based molecular studies

Hitherto, only a single attempt has been made to assess genetic relationship between *Jatropha* species based on nuclear (nr) DNA internal transcribed spacer (ITS) region (Pamidimarri et al.

2008). The nrDNA ITS region based sequence data showed size variation of the ITS region across seven *Jatropha* species (*J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica* and *J. tanjorensis*). The nrDNA ITS sequence based dendrogram showed moderate congruence with the dendrograms obtained using multilocus markers system reported earlier.

Intra-specific diversity based studies

Characterization of *J. curcas* germplasm collections from diverse ecogeographical regions of India with the assistance of molecular markers have been carried out by several independent research groups. The earliest report of using RAPD markers to study the difference between toxic Indian variety and non-toxic Mexican variety was by Sujatha et al (2005). The RAPD-based polymorphisms generated in this study served as unique fingerprints to distinguish Mexican non-toxic variety from Indian toxic variety. Following this, Basha and Sujatha (2007) delineated the phylogenetic relationship among the *J. curcas* germplasm collected from various geographic locations in India along with a non-toxic genotype from Mexico using RAPD and ISSR markers, and the study indicated a narrow genetic base in the local germplasm. Ranade et al. (2008) reported studies on the diversity amongst the *J. curcas* accessions; where along with already held collections wild accessions were also included. He inferred that the three wild accessions from North-East India showing highest gene diversity index may contribute more towards widening the genetic diversity within *Jatropha* germplasm. In an attempt to estimate the genetic diversity between diverse provenances of *J. curcas*, Gupta et al. (2008) substantiated the fact that there is only a moderate genetic diversity within accessions from Rajasthan, Uttaranchal, Uttar Pradesh and Orissa. Similar conclusions were also made by Subramanyam et al. (2009) and Pamidimarri et al. (2010) for accessions collected from diverse eco-geographical region. In a similar study, Pamidimarri et al. (2009b) used RAPD and AFLP methods to generate markers specific to non-toxic and toxic accessions of *J. curcas*. The study explicitly established the fact that the non-toxic Mexican accessions (with low phorbol ester content) are genetically different from the toxic Indian accessions. In contradiction to previous studies, Tatikonda et al. (2009) observed higher genetic diversity for accessions from Andhra Pradesh and Chhattisgarh in AFLP-based molecular characterization. However, in an independent investigation, Jubera et al. (2009) reported low percentage of polymorphism among *Jatropha* genotypes from South India. The few accessions and less number of primers used in the study might have failed to delineate the true picture of *J. curcas* diversity. In two subsequent studies, Umamaheswari et al. (2010) reported a moderate amount of diversity while Ikbal et al. (2010) observed high diversity among *J. curcas* genotypes using ISSR and RAPD techniques respectively. However, the major part of this floristically enriched region has not been thoroughly studied hitherto. Thus, regardless of the number of accessions used, most of the aforementioned studies confirmed the existence of only a moderate amount of genetic variation among the *J. curcas* genotypes in India and axiomatically indicated the need for broadening the genetic base of *J. curcas* through introduction of wild provenances from diverse eco-geographical provinces having minimum human interventions.

2.6.3 Phytochemical studies with special reference to seed-toxins

2.6.3.1 Seed phytochemistry and toxicity

J. curcas seeds are rich sources of hydrocarbons and have created surge of interest worldwide for the use of its seed oil as a commercial source of biofuel. The seed oil content ranges from 30 to 50% by seed weight and 45 to 60% by kernel weight (Pramanik 2003). The major fatty acids of *Jatropha* oil are oleic acid (34.3-45.8%; 18:1), linoleic acid (29.0-44.2%; 18:2), palmitic acid (14.1-15.3%; 16:0) and stearic acid (3.7-9.8%; 18:0). The nitrogen and phosphorus rich *J. curcas* seed cake left after oil extraction is potentially of high market value as an animal feed ingredient and organic fertilizer (Augustus et al. 2002; Costa et al. 2010). Extraction of per kilogram of oil from *Jatropha* seeds results in production of about 0.75 kg of seed kernel meal as a byproduct. Despite having a high protein content (~65% on a dry matter basis) with essential amino acids, the kernel meal is not used for animal feed because of the presence of high concentrations of anti-nutritional factors (ANFs) such as saponins, lectins, curcins, phytate, protease inhibitors and phorbol esters. The high concentrations of phorbol esters, a group of tetraylic diterpenoids, present in *J. curcas* kernels has been identified as the main toxic and co-carcinogenic agent responsible for *J. curcas* toxicity (Francis et al. 2013). Methods to detoxify the seed cake have been proposed (Devappa and Swamylingappa 2008). However, the co-carcinogenic phorbol esters are not destroyed by easy and cost-effective conventional heat treatment methods. The difficulty of removing phorbol esters not only from the seed cake but also from the oil and even from the biodiesel itself, confines the commercial exploitation of *J. curcas* and its co-products, despite the high nutrient content.

2.6.3.2 Phytochemical component (phorbol ester) based diversity studies in *Jatropha*

Few studies have been reported on the estimation of phorbol ester content of *J. curcas* seeds collected from diverse locations for identification of elite accessions having low concentration of phorbol ester. Makkar et al. (1998) reported the presence of phorbol ester in negligible amount in Mexican varieties of *J. curcas*, when compared with the varieties obtained from Cape Verde and Nicaragua. In a recent study, He et al. (2011) compared phorbol ester content of 39 *Jatropha curcas* seed samples obtained from Madagascar (23 samples), Mexico (14 samples), Tanzania (1 sample) and Suriname (1 sample). He suggested that based on phorbol ester content two classes of seeds are available, one with high concentration and the other at least 1000 times lower.

2.6.4 Correlation of morphometric, molecular and phytochemical diversity in *Jatropha*

Establishment of the correlation between morphometric traits, phytochemical characters and molecular fingerprinting data has become a prerequisite for generation of functional markers which will assist in marker assisted genetic improvement of *J. curcas*. However, the majority of the *Jatropha* diversity studies have either utilized morphological or molecular markers. To date, very few correlation studies have been conducted in *J. curcas*. The first comprehensive attempt to assess the genetic relationship between *J. curcas* genetic resources from thirteen countries on the basis of biochemical characteristics (seed kernel protein content, oil content, ash content and phorbol ester content) and molecular markers (RAPD, ISSR, SSR and curcin-specific markers) was performed by Basha et al. (2009). The study reported high genetic diversity in Mexican germplasm and low genetic variation in accessions from other countries both in terms of phorbol ester levels and molecular profiles. In a subsequent study, Sunil et al. (2011) studied the correlation between the phenotypic and genotypic diversity of 34 *J. curcas* accessions collected from diverse ecogeographical region of India using phenotypic traits (plant height, canopy spread, collar length, primary branches, petiole length, pedicel length, fruit clusters, fruits per plant and % oil content) and molecular markers (RAPD and ISSR) and concluded that more corroborative studies are required for identification of elite accessions for *J. curcas* breeding programs. However, the study failed to comprise accessions from diverse regions of East and North-East India. Thus, it can be conferred that further studies in *J. curcas* germplasm characterization should be encouraged with the aim of bridging linkage between morphometric, molecular and biochemical data.

2.6.5 North-East region of India: hotspot of biodiversity

North-East India, a part of both trans-Himalaya and Indo-Burma biodiversity hotspots, is gifted with ecological diversity (Myers et al. 2000; Pawar et al. 2007). North-East India lies between 29.46°– 21.96°N and 97.39°–89.87°E covering seven sister states of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland and Tripura. The region has been sub-divided into three biogeographic zones (Fig. 2.3) – (1) Eastern Himalayas in the north (Arunachal Pradesh), (2) the Northeast Hills in the south (Nagaland, Manipur, Meghalaya and Mizoram) and (3) the Brahmaputra River Basin in between (Assam and Tripura) (Pawar et al. 2007). The climate of this region varies from tropical to temperate and altitude varies from 50 m in tropical foothills to 7000 m in alpine zones. Average rainfall ranges between 800 mm and 4000 mm (Sajeev et al. 2011). The geographic location, high rainfall, diverse climatic condition and limited human intervention make this region richest plant diversity reservoir of India. The species *J. curcas* is now well naturalized and has become common flora in this unique bio geographic region (Mazumdar et al. 2012).

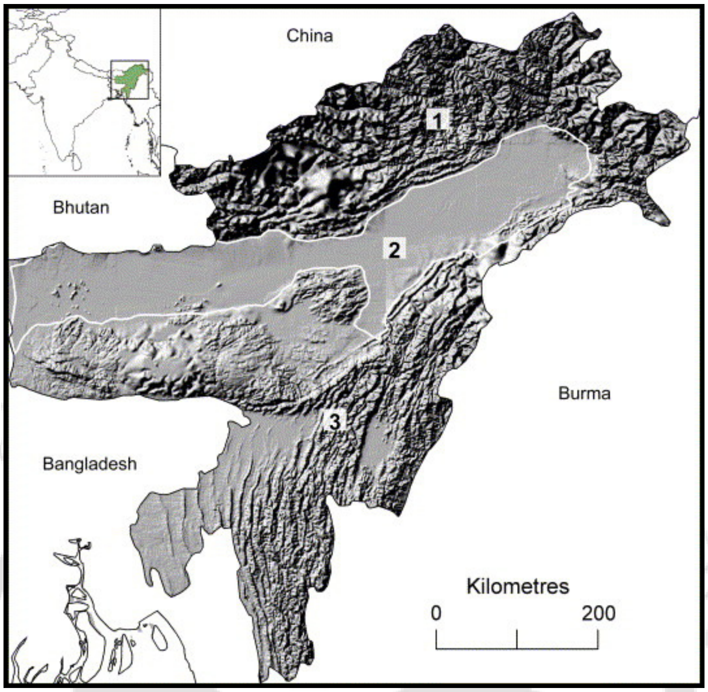


Fig. 2.3 Digital elevation model of North-East India with major subregions - (1) Eastern Himalaya, (2) Brahmaputra River Basin and (3) North-East Hills (Pawar et al. 2007)

However, little research has been conducted on morphometric characterization of *J. curcas* germplasm from North-East India (Saikia et al. 2009). In an attempt to assess the molecular variation of North-East accessions of *J. curcas*, Kumar et al. (2011) recently has reported the genetic diversity analysis of *Jatropha* germplasm covering only two states (Assam and Meghalaya) of North-East India using very few primers for the investigation. However, the major part of this floristically enriched region has not been thoroughly studied hitherto. An associative study complementing morphometric, molecular and biochemical characterization of *J. curcas* germplasm from agroclimatically diverse regions of North-East India may facilitate in identification of diverse and elite genotypes which can further be efficaciously exploited in *Jatropha* improvement programs.

A comprehensive list of major morphometric, molecular and biochemical characterization studies carried out to date in *Jatropha* has been consolidated in Table 2.1a and 2.1b.

Table 2.1.a - A consolidated list of morphometric, molecular and phytochemical analyses based worldwide diversity studies in *Jatropha*

Sl. No.	Authors	Markers /traits/ parameters	Collection site	No. of accessions	Diversity
1	Hailu (2010)	**Morphometric:** Growth characters, Leaf and Seed traits, **Molecular:** ISSR (5 primers) **Biochemial:** Seed oil content	Ethiopia	11	High
2	Santoso (2011)	**Morphometric:** seed traits, seedling characteristics **Biochemical:** Kernel oil content	Indonesia	5	High
3	Unanaonwi and Nwabueze (2012)	**Morphometric:** Growth characters	Nigeria	4	High
4	Yang et al. (2010)	**Morphometric:** Growth characters, Seed traits **Biochemial:** Seed oil content	South China	80	High
5	Shabanimofrad et al. (2013)	**Morphometric:** Growth characters, Leaf traits, Seed traits **Biochemical:** Seed oil content	Malayasia	48	High
6	Montes et al. (2008)	**Molecular**	Asia, Africa, Latin America	225	High: Latin America Low: Asia, Africa
7	Sun et al. (2008)	**Molecular:** SSR, AFLP	South China	58	Low
8	Cai et al. (2010)	**Molecular:** ISSR **Biochemical:** Kernel oil content	South China, Myanmar	224	High
9	Basha et al. (2009)	**Molecular:** RAPD, ISSR, SSR, curcin-specific primer **Biochemical:** Crude protein, Oil content, Ash, Phorbol ester	India (19),Cape Verde (1), Mexico (28), Madagascar (9), El Salvador(1), Uganda (3), Africa (1), Egypt (1), Vietnam (1), China (4), Malayasia (1), Philippines (2), Thailand (1)	72	High: Mexico (both biochemical analyses based on phorbol ester content and molecular diversity)
10	Shen et al. (2010)	**Molecular:** AFLP	China and Indonesia	38	Low

Sl. No.	Authors	Markers /traits/ parameters	Collection site	No. of accessions	Diversity
11	Shen et al. (2012)	**Molecular:** AFLP	China (27), Indonesia (1), Laos (2), India (17), Mali (3), Mexico (5), Papua New Guinea (1), Philippines (2), Thailand (2), Vietnam (3)	63	High: Mexico
12	Grativol et al. (2011)	**Molecular:** ISSR	Brazil	332	Moderately high
13	Pecina-Quintero et al. (2011)	**Molecular:** AFLP	Mexico	88	High
14	Makkar et al. (1998)	**Biochemical:** Anti nutritional factors, Amino acid, Crude protein, Lipid, Ash	Nicaragua, Nigeria, Mexico	4	Phorbol ester : negligible in Mexican variety
15	He et al. (2011)	**Molecular:** AFLP **Biochemical:** Phorbol ester, Curcin	Madagascar, Mexico	39	Madagascar: Low Diversity Mexico: High Diversity, Phorbol ester content negligible

Table 2.1.b - A consolidated list of morphometric, molecular and phytochemical analyses based diversity studies in *Jatropha* in India

Sl. No.	Authors	Markers /traits/ parameters	Collection site	No. of accessions	Diversity
1	Ginwal et al. (2004)	**Morphometric:** Growth characters, Seed traits (Quantitative) **Biochemical:** Oil content	Madhya Pradesh and Maharashtra	10	Moderately high
2	Ginwal et al. (2005)	**Morphometric:** Growth characters, Seed traits (Qualitative and Quantitative) **Biochemical:** Oil content	Madhya Pradesh and Maharashtra	10	Moderately high
3	Kaushik et al. (2007)	**Morphometric:** Seed traits **Biochemical:** Oil content	Haryana	24	High
4	Rao et al. (2008)	**Morphometric:** Growth characters, Seed traits **Biochemical:** Oil content	Andhra Pradesh	32	High
5	Das et al. (2008)	**Morphometric:** Growth characters	Gujarat, Orissa, Rajasthan	18	Moderate
6	Sunil et al. (2008)	**Morphometric:** Growth characters **Biochemical:** Oil content	Chhattisgarh and Andhra Pradesh	162	High
7	Sunil et al. (2009)	**Morphometric:** Growth characters **Biochemical:** Oil content	South east coastal zone	100	High
8	Gohil and Pandya (2009)	**Morphometric:** Growth characters, Seed traits **Biochemical:** Oil content	Gujarat and Rajasthan	7 (toxic), 2 (non toxic)	High

Sl. No.	Authors	Markers /traits/ parameters	Collection site	No. of accessions	Diversity
9	Saikia et al. (2009)	**Morphometric:** Growth characters, Seed traits **Physiological:** Photosynthetic rates, stomatal conductance	Gujarat, Orissa, Andhra Pradesh, Haryana, Uttarakhand, Manipur, Arunachal Pradesh, Nagaland, Tripura, Meghalaya, Mizoram, Assam, West Bengal, Tamil Nadu, Kerala, Jharkhand	34	Growth performance consistent for Arunachal, Pradesh, Assam, Manipur and Nagaland accessions
10	Mohapatra and Panda (2010)	**Morphometric:** Growth characters, Seed traits **Biochemical:** Oil content	Diverse locations of India	20	High
11	Pandey et al. (2010)	**Morphometric:** Growth characters	Madhya Pradesh	20	7 diverse accessions were screened
12	Ghosh and Singh (2011)	**Morphometric:** Seed and seedling traits	Western Himalaya, Sutlej Ganga plains, eastern and south eastern uplands, western plain, central highlands, western Ghats	32	4 candidate plus plants were screened
13	Gairola et al. (2011)	**Morphometric:** Seed traits	Uttarakhand Himalaya	11	Moderate
14	Naresh et al. (2012)	**Morphometric:** Growth characters (Qualitative and Quantitative), Seed traits **Biochemical:** Oil content	Andhra Pradesh and Rajasthan	14	High
15	Kumar et al. (2008)	**Molecular:** ISSR	Andhra Pradesh, Tamil Nadu, Rajasthan, Zimbabwe	8 *Jatropha* species + 3 *J. curcas* accessions	Highest genetic diversity with *J. curcas*: *J. integerrima*
16	Ram et al. (2007)	**Molecular:** RAPD	Andhra Pradesh and Tamil Nadu	7 *Jatropha* species + 5 *J. curcas* accessions	Highest genetic diversity with *J. glandulifera*
17	Vijayanand et al. (2009)	**Morphometric:** Growth characters, Leaf traits **Molecular:** ISSR	Andhra Pradesh and Tamil Nadu	7 *Jatropha* species + 5 *J. curcas* accessions	High
18	Basha and Sujatha (2009)	**Molecular:** RAPD, ISSR, chloroplast specific markers	Andhra Pradesh and Tamil Nadu	10 *Jatropha* species	Highest genetic diversity with *J. podagrica*, *J. multifida*

Sl. No.	Authors	Markers /traits/ parameters	Collection site	No. of accessions	Diversity
19	Pamidimarri et al. (2009)	**Molecular:** ITS specificmarker	Gujarat	7 *Jatropha* species	Highest genetic diversity with *J. multifida*
20	Sujatha et al. (2005)	**Molecular:** RAPD **Biochemical:** Phorbol ester	India (toxic), Cape Verde (toxic), Mexico (non toxic)	3	High
21	Basha and Sujatha (2007)	**Molecular:** RAPD, ISSR	Tamil Nadu, Kerala, Andhra Pradesh, Madhya Pradesh, Haryana, Rajasthan, Mexico	43	Narrow diversity in Indian accessions
22	Ranade et al. (2008)	**Molecular:** RAPD,DAMD	Orissa, Uttaranchal, Uttar Pradesh, Sikkim, Arunachal Pradesh, Meghalaya	21 (*J. curcas*) + 2 (outgroup)	High Diversity: Sikkim, Arunachal Pradesh, Meghalaya accessions
23	Gupta et al. (2008)	**Molecular:** RAPD, ISSR	Orissa, Rajasthan, Uttaranchal, Uttar Pradesh	13	Moderate
24	Subramanyam et al. (2009)	**Molecular:** RAPD	Andhra Pradesh, Karnataka, Orissa	10	Moderate
25	Pamidimarri et al. (2010)	**Molecular:** RAPD, AFLP	16 diverse provenances	28	Low
26	Pamidimarri et al. (2009)	**Molecular:** RAPD, AFLP, SSR	India, Mexico	6 (toxic), 1 (non toxic)	High
27	Tatikonda et al. (2009)	**Molecular:** AFLP	Andhra Pradesh, Chhattisgarh, Gujarat, Madhya Pradesh, Rajasthan, Uttar Pradesh	48	High diversity: Andhra Pradesh and Chhattisgarh accessions
28	Jubera et al. (2010)	**Molecular:** RAPD	Madhya Pradesh, Maharashtra, Karnataka, Tamil Nadu	7	Low
29	Umamaheswari et al. (2010)	**Molecular:** ISSR	Tamil Nadu, Zimbabwe (1)	17	Moderate
30	Ikbal et al. (2010)	**Molecular:** RAPD	Gujarat, Haryana, Madhya Pradesh, Punjab, Rajasthan	40	High
31	Sunil et al. (2011)	**Morphometric:** Seed traits **Molecular:** RAPD, ISSR **Biochemical:** Oil content	Andhra Pradesh, Chhattisgarh, Rajasthan, Uttarakhand	34	High
32	Kumar et al. (2011)	**Molecular:** DAMD, ISSR **Biochemical:** Oil content	Assam, Meghalaya	6	High
33	Kumar et al. (2013)	**Molecular:** DAMD, RAPD	Assam, Meghalaya	6	High

2.7 Current objectives of *Jatropha* genetic improvement

Despite the several benefits associated with *J. curcas* cultivation, few serious apprehensions are associated with acceptance of this plant as a potential biodiesel feedstock. Narrow diversity in germplasm, limited information about *J. curcas* genome, inconsistent seed yield, unpredictable oil content and seed poisoning are the major concerns. Identification of superior genotypes of *J. curcas* having superior agronomic traits along with establishment of *J. curcas* genome database may help in overcoming these constraints. The current objectives of *J. curcas* improvement should aim at higher seed yield and oil content, improved biodiesel properties, earlier maturity, reduced plant height, resistance to pests and diseases, abiotic stress tolerance and higher ratio of female to male flowers.

2.8 Approaches for *Jatropha* improvement

2.8.1 Broadening diversity in germplasm for identification of superior genotypes

The local and global investigation of genetic diversity of *J. curcas* genotypes, as conferred earlier, revealed a narrow diversity in the germplasm. For reducing redundancy in *J. curcas* germplasm due to inbreeding depression, the collection and conservation of diverse populations from wild habitat before it gets extirpated due to human interference is of utmost importance. The primary reason given for the prioritization of germplasm collection is for inclusion and conservation of elite varieties with economically significant traits like high seed yield and oil content, high female to male flower ratio, disease resistance, continuous flowering and fruiting, synchronous fruit ripening and so forth. Establishment of a network program at international level on global *J. curcas* genetic resource to ensure the conservation of diverse and elite genotypes across the globe along with availability and sustainable utilization of the *J. curcas* genotypes for tree improvement programs is of immense significance. In India, the National Bureau of Plant Genetic Resources (NBPGR) (www.nbpgr.ernet.in) has been assigned with the responsibility of maintenance and conservation of superior plant genotypes. Six diverse accessions of *J. curcas* having significant variation in flower trait, fruit trait and high oil content has been registered in NBPGR (Table. 2.2). Furthermore, the National Oilseeds and

Vegetable Oils Development (NOVOD) Board (Ministry of Agriculture, Government of India) (www.novodboard.com) along with other institutions has initiated a nationwide network program on Tree Borne Oilseeds with the objective of identification of Candidate Plus Trees (CPTs) and development of superior varieties/hybrids of *Jatropha* and other oilseed plants. Superior CPTs of *Jatropha* for seed yield, oil content, plant height, number of branches/plant, collar diameter, low temperature tolerance and disease resistance have already been identified (Table. 2.2) [1]. Future research in *J. curcas* should target identification of elite plants having important agronomic traits like consistently high female to male flower ratio, synchronous fruit maturation and seed ripening, pest resistance and low toxin content.

Table 2.2 - A consolidated list of Candidate Plus *Jatropha* plants identified in India

Sl. No.	Unique features	National Identity Number
***Jatropha* germplasm registered for specific trait(s) with NBPGR, India**		
1	High oil content	IC541650 (40.6%), IC537939 (42%)
2	Brownish purple fruit	IC566227
3	Oblong fruit	IC566228
4	Pink flower with continuous flowering	IC427819
5	White flower with continuous flowering	IC427820
Sl. No.	**Unique features**	**Name of genotypes**
Candidate Plus *Jatropha* Trees identified by NOVOD, India		
1	High seed yield	RJ- 117, TNMC- 22, TFRI -2, Pant J Sel -1, TFRI-1 etc.
2	High oil-content	TFRI 01 (49.2%), MNJ-017(42.89 %), TNMC-22 (39.43%), PDKV-2 (39.07%), IGAU-1 (37.61%) etc.
3	Branches/plant	TFRI-04 (49.73), TFRI-02 (49.49), PJ-02 (48.97) etc.
4	Plant height	JIP-15, RJ-117, PJ-01 etc.
5	Collar diameter	TFRI-03 (109.82 mm), PJ-01 ((102.69 mm) etc.
6	Frost/low temperature tolerant	JR-17, Jwalaji Local
7	Powdery mildew resistant	MNJ-001, JIP-13, PJ-01 and PJ-02
8	Viral disease resistant	JIP-13, JIP-15, TFRI-03 and TFRI-04

2.8.2 Mapping and Sequencing of *Jatropha* genome

The advent of DNA-based genetic markers has facilitated efficient prediction and tagging of important quantitative trait loci (QTLs) linked to major economic and agronomic traits in many plants. This has eventually helped in marker based molecular mapping of plant genome as well as map based cloning and plant breeding. The identification of QTLs associated with seed yield potential, phorbol esters and seed-oil content via linkage to molecular markers is primordial for genomics based breeding strategies in *J. curcas* that will subsequently help in development of candidate plus varieties with high seed yield potentials, oil content and low toxin content. The fact that *Jatropha* genome is of relatively small size (416 Mb) suggests that it is ideally suited for genome mapping, sequencing and identification of candidate genes (Fu et al. 2011). However, the genetic map of *J. curcas* is not yet well-developed and very limited information is available on its genetics and genomics. Recently, Asif et al. (2010) has determined the complete nucleotide sequence of *J. curcas* chloroplast genome using pyrosequencing along with Sanger sequencing while Sato et al. (2011) has reported the whole genome sequence of *J. curcas* using a complementation of Sanger's method and new-generation multiple sequencing methods Recently, some genes and QTLs related to important agronomic traits have also been reported for *J. curcas* (Liu et al. 2011, Wang et al. 2011). Therefore, availability of genome sequences along with discovery of agronomic trait linked loci and genes have made construction of supersaturated genetic map of *J. curcas* feasible. This will later facilitate genome-wide tracking and efficient cloning of genes and QTLs having agronomic significance for the development of elite varieties [2].

2.8.3 Increase in female flower number and total seed yield

Female to male flower ratio is an important yield determinant in *J. curcas*. However, the number of female flowers in *J. curcas* is much less in comparison to the number of male flowers. Increase in number of female flowers will subsequently increase the total fruit and seed yield in *J. curcas*. Attempts to increase number female flowers in inflorescence by application of plant growth regulators, 6-benzyladenine and paclobutrazol, resulted in increase of female to male flower ratio in *J. curcas* with subsequent increase in total fruit and seed yield.

Paclobutrazol treatment also resulted in reduced vegetative growth of the plant along with stunted height (Pan and Zu 2011; Ghosh et al. 2010.). Application of gibberellin in low concentration resulted in alteration of sex ratio with formation of more female flowers (Makwana et al. 2010). Tissue culture based approaches like large scale micropropagation of *J. curcas* using elite genotypes having high seed yield with subsequent assessment of genetic fidelity of plantlets with candidate plus mother plant may facilitate in overcoming the barriers of inconsistent seed yield (Sharma et al. 2011).

It has been observed that in many plant species, ethylene plays a significant role in sex determination by increasing the number of pistillate flowers (Yamasaki et al. 2003; Papadopoulou et al. 2005). Modification of floral sex ratio via transgenic approaches in *Jatropha* will primarily aim on regulation of ethylene biosynthesis genes. Alterations in the floral homeotic genes in *J. curcas* may also lead to the changes in floral organ. In soybean, an important oilseed plant, it was found that *GmAP1, an APETALA1*-like gene regulates flowering time and floral organ specification while in Arabidopsis, *APETALA2* has an significant role in determining total seed yield (Chi et al. 2011; Jofuku et al. 2005).

In some plants, environmental factors like photoperiod and temperature have direct influence in floral sex ratio (Thomas 2008). In *Jatropha*, it has been reported that temperature changes have direct influence on male to female flower ratio of *Jatropha* (Fresnedo-Ramirez 2013). An extreme temperature fluctuation induces early abortion in the male flowers and inhibits opening for the female flowers during day time. Recently, three floral identity genes (*APETALA2, APETALA3 and PISTILLATA)* along with five flowering regulators *(CONSTANS, FLOWERING LOCUS D, FLOWERING LOCUS F, LEAFY* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1) has been identified in Jatropha genome. CONSTANS* plays a significant in the regulation of flowering by photoperiod in rice and barley (Fresnedo-Ramirez 2013). Dehgan and Webster (1979) previously have suggested that the floral sex ratio of *J. curcas* could be altered by photoperiod. However extensive survey of literature confirmed the fact that until now, no experiments has been carried out to determine the effect of photoperiod on the male to female flower ratio of *Jatropha*. The identification of *CONSTANS* in *Jatropha* genome may help in future to understand the influence of photoperiod in *J. curcas* floral sex ratio.

Regulation of expression of these flowering-related genes in *J. curcas* via genetic engineering may result in improvement of floral sex ratio and seed yield potential. Transformation protocols of *J. curcas* for *Agrobacterium tumefaciens* mediated gene transfer and particle bombardment have already been developed (Mazumdar et al. 2010; Kumar et al. 2010; Misra et al. 2012; Purkayastha et al. 2010; Joshi et al. 2011). Molecular breeding and hybridization can serve as alternative means to increase female flower, total fruit and seed yield in *Jatropha*. Nandan-1, a high seed yielding hybrid, has recently been successfully developed by crossing two *J. curcas* parents, JCZ-I84 (♂), having high number of inflorescences per plant, and JCZ-P34 (♀), having high number of female flowers, respectively (Karanam and Bhabanasi 2009a). Interspecific hybridization using *J. integerrima* as one of the parents followed by backcrossing with *J. curcas* can lead to the introgression of the desired gene for high female flower in the backcrossed populations. The QTLs controlling female flower number, fruit number and seed yield traits in *Jatropha* has already been located (Sun et al. 2012).

2.8.4 Synchronous flowering and fruiting

Due to asynchronized flowering period in *J. curcas*, the fruits ripen throughout the year in successive phases. However, for facilitating mechanical and manual fruit harvesting, induction of synchronous flowering and fruit maturity along with specific harvesting period is of immense importance. It has been reported that chemical defoliants like maleic hydrazide (MH) can induce synchronized anthesis in fruit crops in tropical regions (Atkinson et al. 2013). Moreover, MH is an important chemical agent for increasing total number of flowers in *J. curcas* (Abdelgadir et al. 2010). Systematic study of effect of MH on synchronous flowering of *J. curcas* may provide useful information on harmonizing flowering period in this plant. Circadian clock regulated genes also plays a crucial role in modulation of flowering time by photoperiod perception in plants. Recently, functional characterization of circadian clock regulated DNA-binding transcription factors with one finger genes, *JcDof1* and *JcDof3*, from *J. curcas* suggested that these genes might play important roles in regulation of flowering time of *J. curcas.* *JcDof1* is a light-dependent circadian-clock gene while *JcDof3* acts as a negative regulator in the photoperiodic flowering pathway of *Jatropha* (Yang et al. 2010b; Yang et al. 2011). The role of MADS box genes in modulation of flowering times has already been well documented. Recently, five paralogs of *SHORT VEGETATIVE PHASE* (*SVP),* a flowering time controlling gene of the MADS box family, have been identified in *Jatropha* (Sato et al. 2011). Regulation of these genes may facilitate in improvement of circadian rhythms along with promotion of synchronous flowering and fruit ripening in *J. curcas* via genetic engineering.

2.8.5 Enhancement in seed oil content

The major impediment behind large scale cultivation of *J. curcas* as an energy crop is low or unpredictable seed oil content. Increment of seed weight and subsequently seed oil content by the application of plant growth regulators can be a feasible approach to increase economic yield of *Jatropha*. Paclobutrazol (PB), a synthetic plant growth regulator, helps in increasing seed weight and oil content in *Jatropha* (Ghosh et al. 2010). Similar results due to application of PB have also been observed in another non-food oilseed crop, *Camelina sativa* (Kumar et al. 2012). Thus, application of an optimized concentration of PB can help in achievement of higher oil yield from *J. curcas*, which is indispensable for the commercial exploitation of the plant. Enhancement in *J. curcas* seed oil content can also be achieved through transgenic approaches by altering expressions of genes involved in triacylglycerol (TAG) synthesis pathways. In *Jatropha*, unique expressed sequence tags (unigenes) involved in seed development and oil biosynthesis has been identified from developing seeds. These genes may serve as potential resource for transgenic researches in *Jatropha* for modulation of oil composition and increase in seed oil content (Natarajan et al. 2010). Increase in seed oil content can also achieved by overexpression of genes encoding for candidate enzymes involved in TAG accumulation using seed specific promoters. Phospholipid:diacylglycerol acyltransferase (PDAT), Glycerol-3-phosphate acyltransferase (GPAT), Lysophosphatidic acid acyltransferase (LPAAT) and Diacylglycerol acyltransferases (DGAT) are the main enzymes involved in TAG biosynthesis pathway (Napier and Graham 2010). *DGAT1*, an isoform of DGAT, is the candidate gene encoding for the enzyme DGAT1, the final enzyme in Kennedy pathway, which plays a significant role in TAG biosynthesis in *Jatropha* seeds by transferring a fatty acid from the fatty acyl-CoA pool to Diacylglycerol (DAG), yielding CoA and TAG (Xu et al. 2011). PDAT catalyzes formation of TAG in an acyl-CoA independent reaction by transferring sn-2 acyl group from phospholipids to DAG. Overexpression of DGAT1 in other oil-seed crops have resulted in increase of seed-oil content (Taylor et al. 2009; Weselake et al. 2007). Recently, both DGAT and PDAT were found to have major contributions in regulation of TAG accumulation in Arabidopsis seeds (Zhang et al. 2009). Expression patterns of DGAT and PDAT in developing seeds of *Jatropha* have already been delineated (Xu et al. 100). Overexpression of these genes along with other candidate genes of Kennedy pathway through genetic engineering approaches may lead to the development of new transgenic lines having increased seed oil content in *Jatropha*. Alternative approach for increasing TAG content in *Jatropha* seeds can involve regulation of functions of genes involved in biosynthesis of glycerol-3- phosphate (G3P), the initial substrate of Kennedy pathway. Overexpression of a yeast gene coding for the enzyme glycerol-3-phosphate dehydrogenase which catalyzes the formation of G3P has resulted in 40% increase in the final lipid content of *Brassica napus* seeds (Vigeolas et al. 2007). Application of similar strategies in *J. curcas* may result in increase of seed oil content. For successful commercialization of transgenic *J. curcas* and development of new variety, the novel transgenic lines would have to be coupled with the commonly available genotypes through plant breeding. However, traditional breeding approaches for seed related traits is time consuming and unprofitable as attainment of maturity is important for evaluation of the success of breeding and selection of superior varieties. In contrary, application of molecular marker techniques like Marker Assisted Selection (MAS) in *J. curcas* through identification of QTLs related to quantitative traits like seed oil content may provide immense opportunity for selection of promising genotypes at early seedling stages. However due to unavailability of high marker density for agronomically important traits, molecular breeding in *J. curcas* is still in the early stage of development. Recently, plant breeding initiatives for improvement of agronomic traits has resulted in identification of a significant QTL (*qOilC-4*) controlling total oil content in *Jatropha* hybrids CI7041 (*J. curcas* PZM16 x *J. integerrima* S001) and backcrossed populations

(PZM16 x CI7041) (Liu et al. 2011). In a consequent investigation pleiotropic QTLs having crucial roles in growth and seed related characteristics were identified in *Jatropha* hybrids by the same research group. This study also indicated that seed related traits in *Jatropha* are controlled by multiple gene complexes (Sun et al. 2012). Thus, inclusion of the seed trait and oil characteristics related QTLs in MAS will assist in selection of superior varieties of the plant in *J. curcas* improvement programs.

2.8.6 Improvement in biodiesel properties

The composition of *J. curcas* seed oil has not yet been optimized for the production of ideal biodiesel for better engine performances. Alteration of seed fatty acid composition and concentration can augment the suitability of *J. curcas* seed oil as a biodiesel feedstock. Conventionally chemical methods are used for improvement of biodiesel properties. *Jatropha* biodiesel has good cold-flow properties (cloud point and pour point), but is highly susceptible to oxidation. Oxidation stability of *Jatropha* biodiesel can be improved conventionally by doping of methyl esters with stabilizer or antioxidants (phenolic and aminic antioxidants) (Jain and Sharma 2011). Even though it is feasible to reach the desired oxidation stability specification by using doping agents but there will be cost implications as antioxidants are expensive and thus can increase total production cost of *J. curcas* biodiesel. Biotechnological approaches can be exploited effectively for lowering the economics of biodiesel synthesis. Genomics and transgenic technologies provides arsenal of genetic tools for manipulation of genes involved in seed oil biogenesis for production of high concentrations of “designer” oil having favorable fatty acid composition along with desirable biodiesel properties (cetane number, kinematic viscosity, cold-flow, and oxidation stability). Among the significant biodiesel properties, the oxidation stability of biodiesel is directly related to concentration of monounsaturated fatty acids (MUFA). An ideal feedstock for biodiesel should have low concentrations of polyunsaturated fatty acid (PUFA) and high concentrations of MUFA (Ramos et al. 2009). In most of the plants, oleic acid (18:1), a MUFA, is metabolized to linoleic acid (18:2), a PUFA, by a single fatty acid desaturation step catalyzed by a Δ12-desaturase encoded by the *fatty acid desaturases-2* (FAD2) gene (Beló et al. 2008). Recently, genome wide analysis in *J. curcas* has resulted in the identification of three putative Δ12 JcFAD2s genes responsible for the accumulation of high concentrations of polyunsaturated linoleic acid (30-50%). Downregulation of one of these genes (JcFAD2-1) in a seed specific manner through RNA interference resulted in the increase of monounsaturated oleic acid content from 37% to 78% at the expense of PUFA (41% to 3%) in the oil extracted from transgenic *Jatropha* seeds (Qu et al. 2012). However, cold flow properties of *Jatropha* biodiesel are directly proportional to the PUFA concentration. Therefore increasing total unsaturated fatty acid (MUFA and PUFA) concentration in *Jatropha* seeds is a more practicable approach for increasing oxidative stability along with cloud point and pour point of *Jatropha* biodiesel. *FATB1* and *SAD1* genes play critical role in determination of degree of saturation in the fatty acid biosynthesis pathway in *Jatropha. JcFATB1* gene encodes for acyl carrier protein thioesterase. Seed specific overexpression of *JcFATB1* gene in Arabidopsis has resulted in reduced concentration of unsaturated fatty acids and increased concentrations of saturated fatty acids (Wu et al. 2009). Thus *JcFATB1* plays a central role in determination of carbon chain lengths of fatty acids in *Jatropha*. Downregulation of *FATB1* expression in a seed specific manner can result in elevated level of total unsaturated fatty acid with subsequent reduction in level of saturated fatty acids in *Jatropha* seeds. *SAD1,* an isoform of *SAD* gene, encodes for stearoyl-acyl carrier protein desaturase-1 enzyme which catalyzes the desaturation of stearoyl- acyl carrier protein ($C_{18:0}$) to oleoyl- acyl carrier protein ($C_{18:1}$). Thus overexpression of *SAD1* under the transcriptional control of a seed-specific promoter through genetic transformation experiments can generate transgenic Jatropha lines having high oleic acid concentration with no negative effect on PUFA concentration. Therefore, downregulation of *FATB1* expression through RNAi or antisense suppression mediated approaches along with upregulation of *SAD1* through overexpression in a co-ordinate manner may deliver transgenic Jatropha lines having superior cold flow properties and high oxidative stability. Application of molecular breeding tools like MAS and interspecific hybridization can also be a feasible approach for enhancement of Jatropha biodiesel properties. Recently, Nandan-3, a *J. curcas* hybrid having high seed-oil and oleic acid content has been developed by crossing two *J. curcas* parents, JCZ-C59 (♂), having high oleic acid content and JCZ-O39 (♀), having high seed-oil content respectively (Karanam and Bhavanasi 2009b). Identification of significant QTLs controlling stearic acid, oleic acid and linoleic acid compositions in backcrossing populations of *J. curcas* x *J. integerrima* will encourage MAS in *Jatropha* for screening of superior inter-specific hybrids having improved oil traits in the future (Liu et al. 105).

2.8.7 Reduction of seed toxicity

As discussed earlier, *J. curcas* seeds contain antinutritive factors of which phorbol ester is the main toxic agent. Several cases of *J. curcas* toxicosis in humans after accidental ingestion of the seeds have been reported with symptoms of giddiness, vomiting and diarrhoea and in the extreme condition even death has been recorded (Becker and Makkar 1998). Furthermore, the cocarcinogenic seeds can cause health risks for farmers and bioprocess workers (King et al. 2009). Phytochemical characterization of *J. curcas* accessions based on phorbol ester concentration may lead to the identification of elite accessions having low concentrations of toxins in seed. Existence of wild non-toxic variety from Mexico having low or non-detectable levels of phorbol esters in seeds have already been reported (Sosa-Segura et al. 2012). However, introduction of non-toxic genotype from Mexico to Indian climatic conditions may result in poor growth performances along with decrease in total seed yield and oil content. Application of molecular genetics approaches like molecular breeding and genetic transformation may help in overcoming the limitations associated with *Jatropha* toxicity. Functional genomics approaches like virus-induced gene silencing (VIGS) can be used as "a priori" tool for unfolding the function of candidate genes involved in toxin production. Ye et al. has proposed VIGS through agroinoculation of Tobacco Rattle Virus (TRV) vectors in *Jatropha* as a genetic tool of choice for unraveling the function of genes related ANF synthesis. An attempt to trigger VIGS in *Jatropha* for curcin, a toxalbumin, resulted in negligible concentrations of curcin in TRV: curcin infiltrated plants when compared with the control plants (Ye et al. 2009). Similar functional genomics studies can be done for other ANFs including phorbol esters. VIGS approach coupled with molecular breeding or map-based cloning will subsequently help in development of non-toxic lines of *Jatropha*. Non toxic trait of *Jatropha* is a dominant maternal characteristic, thus, molecular breeding approaches involving non toxic Mexican genotype as female parent and toxic Indian genotype as male parent may lead to the development of novel non-toxic F1 varieties suitable for Indian environmental conditions (Sujatha et al. 2005). Recently, transcriptome analysis of developing *J. curcas* seeds revealed presence of all transcripts for the major steps involved with biosynthesis of phorbol ester and type-I ribosome-inactivating protein. The contigs identified can further be used in gene expression studies for unraveling the mode of function of ANF genes (King et al. 2011). Transgenic strategies involving transcriptional gene silencing or RNA interfence mediated post transcriptional gene silencing has been effectively used in food crops for removal of toxins and allergens (Gallo and Sayre 2009). Genetic transformation based similar approaches can serve as alternative pathways for eliminating or substantially reducing the concentration of toxins in *J. curcas* seeds by downregulating the candidate genes involved in ANF synthesis. The fact that biodiesel production from *Jatropha* is highly profitable only when the by-products generated during biodiesel production can be sold as valuable products has prioritized the urgency of developing non-toxic *J. curcas* which will subsequently lead to the suppression of cancer potentiating diterpenes and other ANFs from the byproducts and the potential utilization of the latter (Foidl and Eder 1997).

2.8.8 Improvement in plant architecture

J. curcas plant normally attains a height of about 3 to 5 m, but under suitable growth conditions can even reach a height of about 10 m (Divakara et al. 2010). Since *Jatropha* seeds are harvested manually at maturity, greater plant height may result in difficulties while harvesting with subsequent increase in labor cost. Thus reduced plant height, a desirable agronomic trait, facilitates manual harvesting along with an improvement in harvest index (ratio of seed yield to total above-ground biomass). In an attempt to modify plant architecture, overexpression of KN1 gene in *J. curcas* resulted in generation of transgenic lines having dwarf shoots, reduced plant size and alterations of leaf shape (HongJie et al. 2010). Since seed yield in *Jatropha* has significant positive genotypic and phenotypic correlation with the number of branches, increase in ramification of branches can ultimately result in increase in total seed yield (Rao et al. 2008). Commonly, pruning of apical buds of the main stem is used for increasing number of lateral branches through inhibition of apical dominance (Kureel 2006). However, application of chemical agent like plant growth regulators (PGRs) for promotion of branching is much more efficient and cost-effective. Among all PGRs, maleic hydrazide was found to be most efficient in *Jatropha* for increasing ramification of branches (Abdelgadir et al. 2009). However, the effects of these traditional methods are temporary and not carried over to the next generations. Fortunately, in recent years, transgenic researches highlighted the possibility of engineering the extent of shoot branching by upregulation or downregulation of several branch-regulating genes. Furthermore, the expression of transgenes is stable in subsequent generations of the plant. Insertion or over-expression of branch-regulating genes (lateral shoot-inducing factor, isopentenyl transferase etc.) in several plant species including pitunia, kiwifruit etc. resulted in generation of dwarfed and branched transgenic lines (Nakagawa et al 2005; Honda et al. 2011). In a recent study, insertion of petunia lateral shoot-inducing factor gene in *Jatropha* resulted in stable expression of the transgene in *J.curcas* transgenic lines. However, the effect of transgene on lateral branching of transformed plants is yet to study (Zong et al. 2010).

2.8.9 Resistance to biotic stress

J. curcas is considered to be a hardy and disease resistant plant due to the insecticidal, molluscicidal, fungicidal and nematicidal properties of the seed oil. However, in discordance with common belief, several studies have confirmed the fact that *J. curcas* is vulnerable to multitudes of biotic stresses like insect infestation, fungal and viral diseases which ominously contribute to the loss in economic yield of the plant along with degradation of the quality of the seed. *Jatropha* is susceptible to 60 pest species in 21 insect families, 35 fungal species, 4 viral and 4 bacterial pathogens respectively (Fitt 2011; Anitha and Varaprasad 2012). Hemidepterian insects like *Pachycoris klugii* Burmeister, *Agonosoma trilineatum* (seed feeder) and *Scutellera nobilis* Fabr. (causes flower fall, fruit abortion and malformation of seeds) along with lepidopterian *Pempelia morosalis* (inflorescence and capsule borer) are the major insect pests causing noticeable damage in *Jatropha* plantation (Shanker and Dhyani 2006). The plant is prone to different fungal diseases like root rot, collar rot, seed and seedling rot, powdery mildew, downy mildew etc. while the viral attacks in *Jatropha* results in different mosaic diseases (Anitha and Varaprasad). Though *Jatropha* is resistant to most of the common nematodal diseases, the plant pathogenic nematode *Aphelenchoides besseyi* can infect *J. curcas* (Tuan et al. 2009). Maintenance of insect and disease free *Jatropha* plantation is of utmost importance for getting good quality of seeds. Propagation of tissue culture generated virus free healthy planting material in commercial scale may help in development of good quality of *Jatropha* plants. Conventionally, various herbal (neem, vitex, and *Calotropis* based pesticides) and/or chemical (monocrotophos, endosulfan, acephate, indoxacarb, cypermethrin etc.) insecticides and pesticides are used for controlling pest infestation and diseases in *Jatropha* (Gour 2006; Rao et al. 2010). However, the indiscriminant use of chemicals may lead to the emergence of new strains resistant to the chemical controlling agents. Biological control measures, such as application of parasitoids, can serve as an effective method for insect control in *Jatropha*. *Pseudotelenomus pachycor* (egg parasitoid of *Pachycoris klugii*), *Telenomus spodoptera* (egg parasitoid of *Spodoptera litura*), *Bracon hebetor* and *Tachinid sp.* (parasitoids of *Pempelia morosalis) etc.* can be efficiently used for major pest resistance in *Jatropha* (Shanker and Dhyani 2006; Contran et al. 2013). Application of genetic engineering for development of insect and disease resistant transgenic *Jatropha* can provide undeniable potentialities in disease management programs along with minimizing the use of insecticides and pesticides for pest control. *Bacillus thuringiensis* (*Bt*) genes isolated from soil bacterium *Bacillus thuringiensis* is the most widely accepted insect resistant genes. This group of genes confers resistance against major lepidopteran and coleopteran insects by releasing insecticidal crystalline (Cry) proteins and vegetative insecticidal proteins (VIPs). *Cry* genes have been efficiently transferred in important oilseed plants like *Brassica*, Castor and Oil palm for the development of insect resistant transgenic lines (Cao et al. 2008; Sujatha et al. 2009; Lee et al. 2006). In our lab stable transgenic lines of *J. curcas* harboring *Cry1Ac* gene conferring resistance against *Spodoptera litura*, a lepidopteran leaf defoliator, has been successfully developed (data unpublished). Development of transgenic *J. curcas* lines constitutively expressing combinations of *Cry* genes through gene stacking may facilitate in broadening the insecticidal spectrum of the plant in future. However, the effect Cry toxins are not equally lethal for all *Jatropha* pests. Therefore, transgenic expression of other insect resistant genes like *α-amylase inhibitors*, *protease inhibitors*, *avidin* and *lectin* in *J. curcas* may facilitate resistivity against a broad range of insect pests which are not susceptible to Cry toxins. Regulation of defense signaling genes involving ethylene, jasmonate and salicylate can provide a holistic approach to achieve broad spectrum resistance in *Jatropha*. Expression of the master switch gene, non-expressor pathogen related protein 1 (NPR1), in many plant species has resulted in enhanced broad spectrum resistance against several pathogens (Lin et al. 2004; Parkhi et al. 2010). Though *Jatropha* is susceptible to mosaic diseases, but transgenic expression of *cur2p* (coding premature curcin 2 proteins) gene, a type 1 RIP gene from *J. curcas* in tobacco has resulted in increase of resistance against Tobacco Mosaic diseases and some fungal diseases in the transgenic tobacco lines (Huang et al. 2008). Overexpression of *cur2p* or co-expression of the gene along with other antipathogenic genes like *chitinases* and *β-1,3-glucanases* in *Jatropha* can provide an efficient impediment against major viral and fungal diseases like mosaic diseases, mildew, wilt and leaf spot. Recently, identification of 4 EST-based contigs coding for disease resistance protein RPM1 (Contig 4), Indole-3-acetic acid-amido synthetase GH3.3 (Contig 6), ccr4-associated factor (Contig 18) and Ethylene-responsive transcription factor (Contig 33) respectively from developing seeds of *Jatropha* has widened the scope for genetic manipulation of the plant for development of disease resistant varieties (Natarajan et al. 2010). Along with genetic engineering, trait-based plant breeding strategies like interspecific hybridization and marker assisted selection for augmenting disease resistance/tolerance in *Jatropha* can play crucial role in *Jatropha* improvement program. An attempt of interspecific breeding of *J. curcas* as the pollen parent with *J. gossypifolia* as the female parent has resulted in the development of the hybrid (Nandan-4) with superior and unique agronomic characteristics in comparison to the parental lines. The plant is highly resistant to diseases (Karanam and Bhavanasi 2009c). MAS mediated novel breeding approaches for identification of superior alleles of disease and pathogen resistant genes in elite *Jatropha* accessions with subsequent introgression of the gene in the plant will facilitate in improvement of the plant. Recently germplasm screening of *J. curcas* against disease conditions and pathogen attacks lead to the identification of superior accessions in ICAR Research Complex for North Eastern Hill Region, Manipur, India (JIP-13 & -15, TFRI-03 & -04, MNJ-001, PJ-01 & -02) and Federal University of Viçosa, Brazil (UFVJC 72) (Evaristo et al. 2013). The disease and pathogen resistant

J. curcas accessions can be introduced into *Jatropha* breeding programs for enabling successful mapping of disease resistant loci. Efforts on MAS in *Jatropha* for disease and pest resistance in future should initially focus on (i) development of molecular markers such as expressed sequence tag (EST), sequence tagged microsatellites (STMS) and single nucleotide polymorphism (SNP) associated with disease and pest resistance (ii) saturation of genomic region of interest with markers and lastly (iii) accurate mapping of target genes and QTLs conferring resistances to pests and diseases. This will ultimately accelerate pyramiding of resistance genes or QTLs for incorporation of pyramided resistance in elite high seed and oil yielding *Jatropha* accessions through molecular breeding, genetic engineering or integrated breeding approaches for the emergence of disease free high yielding *Jatropha* lines.

2.8.10 Resistance to abiotic stress

The altered environmental conditions caused as a consequence of current global climate change is likely to have an adverse contribution on plant growth. Unlike other plants, *Jatropha* is capable of surviving in extreme environmental conditions (Kumar and Sharma 2008). Still, the high-priority abiotic stressors such as drought, soil salinity and extreme temperature conditions (high and low), which enforce water-deficit stress, has negative effect on normal growth and development of *Jatropha* to a certain extent with subsequent reduction in commercial yield of the plant. Apart from major environmental stressors, other adverse abiotic conditions like water logging and presence of chemicals and pollutants in marginal and wastelands selected for *Jatropha* plantation also hampers the healthy growth of the plant. Thus, examination of the response mechanism in *Jatropha* for identification of underlying genes involved in combating and acclimatization of environmental stress conditions has become prerequisite. However, responses to abiotic stresses in plants are multigenic, overlapping and complex. Furthermore, genotype × environment interactions play a significant role in abiotic stress mechanism. Thus, engineering abiotic stress mechanism is a challenging task. For overcoming this impediment, the endeavor for development of *Jatropha* plants with an inbuilt ability to endure and resist abiotic stresses should involve a concerted approach integrating genomic tools like transgenic technology and molecular breeding.

Drought or water-deficit condition is one of the most daunting and unpredictable abiotic threat affecting agricultural productions worldwide. In drought-stricken areas, plants often get exposed to combination of other environmental stresses like heat and salinity synergistically with drought stress (Mittler 2006). However, it has been found that *J. curcas* is capable of surviving in drought-prone arid and semi-arid regions under sub-optimal water supply (Kumar and Sharma 2008). But *Jatropha* plants in semi-arid lands often have poor growth performance with failure to produce economically profitable seed yield. A recent study has confirmed the fact that water-deficit conditions along with heat stress have deleterious effects on the photochemical activity and oxidative metabolism of *J. curcas* (Silva et al. 2010). On the other hand, cultivation of the plant in arable lands is not cost-effective. Thus, development of drought resistant *Jatropha* plants via transgenic or non-transgenic approach seems to be the most viable option at present for mitigating the detrimental effects of drought conditions. In an attempt to improve the drought tolerance in *Jatropha* via genetic engineering, Tsuchimoto et al. (2012) has developed three separate drought resistant transgenic lines in *Jatropha* under the control of constitutively active promoters – (i) the first transgenic line harboring *PPAT* gene, which codes for the enzyme Phosphopantheteine adenylyltransferase that catalyzes the penultimate step in CoA biosynthetic pathway, (ii) the second line overexpressing the putative drought-stress regulating *NF-YB* gene, which encodes for the B subunit of the nuclear factor Y transcription factor and lastly (iii) the third line coexpressing *GSMT* (glycine sarcosine methyltransferase) and *DMT* (dimethylglycine methyltransferase) genes, which encode for enzymes that catalyze production of GB (glycine betaine), a ziwitterionic osmoprotectant, required for fighting against high salt concentration resulted as a consequence of water deficiency. Besides drought conditions, GB helps in fighting against other abiotic factors like chilling, freezing, excess light, heat and oxidative stresses in many plants. The engineered accumulation of GB in chloroplast of transgenic plants due to expression of chloroplast targeted *codA* (choline oxidase) gene has resulted in enhanced tolerance to a broad spectrum of abiotic stresses in comparison to cytosol targeted GB accumulation (Chen and Murata 2008). Chloroplast targeted transgenic expression of GB in *Jatropha* may prove to be efficacious against a plethora of abiotic stresses. Genetic transformation approaches targeting genes coding for stress-inducible transcription factors might help in transcriptional regulation of the expression of several stress-responsive genes with subsequent enhancement of stress tolerance to a broad range of abiotic stressors at the same time. The family of ethylene responsive element binding proteins (EREBP) is mainly implicated in pathogenic and environmental stress responses in plants. Recently, two genes encoding for EREBP family of transcription factors in *J. curcas*, *ERF* (ethylene response factor) and *DREB* (dehydration-responsive element-binding protein) respectively, have been isolated and characterized (Tang et al. 2007; Tang et al. 2011). Both *ERF* and *DREB* are induced by adverse abiotic conditions like drought, salt and cold stresses. Transgenic expression of both *JcERF* and *JcDREB* independently in Arabidopsis resulted in simultaneous enhancement of salt and freezing tolerance. Thus, overexpression of genes encoding for EREBP family of transcription factors in *Jatropha* may result in enhancement of tolerance to multiple abiotic stresses (i.e., drought, salinity and freezing) concurrently.

Development of EST databases with an emphasis on abiotic stress tolerance is one of the most promising approaches for gaining significant knowledge about molecular basis of stress response mechanism in *Jatropha*. Natarajan et al. (2010) has identified abiotic stress tolerant unigenes from developing seeds of *Jatropha*. With an objective to tailor drought- and salt-tolerant *J. curcas* varieties that are capable of producing economically viable fruit yields, Eswaran et al. (2012) generated an EST database from salt stressed roots of *J. curcas* and identified the contigs and singleton sequences which play important role during abiotic stress. The generated EST databases can serve as a rich resource of genetic information required for development of abiotic stress adapted varieties.

Along with conventional breeding, adoption of molecular breeding mediated approaches like mapping of abiotic stress mechanism related QTLs by using stress-linked markers appears to be very promising for genetically dissecting the complex mechanism of abiotic stress response in *Jatropha*. Even though initiation have been taken for identification of markers related to growth, seed and oil yield in *Jatropha*, investigation for identification of stress related markers in *Jatropha* is inadequate hitherto. In an initial attempt to associate molecular markers with abiotic stresses, methylation sensitive amplification polymorphism

(MSAP) markers have been developed to elucidate the effects of salt stress on DNA methylation in *Jatropha* genome. The polymorphic markers generated in this study may assist in identification of potential target genes for biotechnological interventions (Mastan et al. 2012). Exploration of *J. curcas* germplasm for identification of superior abiotic stress (drought, frost, salinity, submergence, heavy metal etc.) tolerant accessions is an immediate first step en route to the future marker-assisted breeding for abiotic stress resistance. Screening of *J. curcas* accessions for frost tolerance has resulted in identification of a frost resistant *J. curcas* genotype (Jwalaji Local) which is capable of resisting frost damage even at an elevation of 1200 m. However, the frost tolerant variety resulted in poor seed yield [1]. Some high altitude *J. curcas* accessions at Xishuangbanna Tropical Botanical Garden, China, showed tolerance to chilling (Zheng et al. 2009). Investigation of these frost/cold tolerant accessions may help in elucidation of the molecular basis of cold adaptation in *Jatropha* along with identification of potential frost responsive QTLs and genes. Marker-assisted introgression of genes and QTLs conferring abiotic stress tolerance from stress resistant accessions into elite high-yielding accessions of *Jatropha* might result in generation of abiotic stress tolerant high-yielding hybrids suitable for persisting in adverse environmental conditions and wastelands. Some success stories of *Jatropha* hybridization for development of abiotic stress tolerant hybrids have already been reported. A drought resistant variety of *J. curcas* (GaoYou CSC63) has already been bred by the Key Laboratory of Bio-resources and Eco-environment at Sichuan University, China (Ying et al. 150). Furthermore, Nandan-4, an environmental stress resistant hybrid has recently been developed through interspecific hybridization (Karanam and Bhavanasi 2009c). Therefore, it can be forecasted that the comprehensive application of biotechnological tools will eventually assist in sustainable improvement of *J. curcas* against plethora of abiotic stress in near future.

2.9 Conclusion and prospective scenarios

Jatropha curcas crude oil is emerging as a prospective biodiesel feedstock in tropical and sub-tropical countries. The engine performance, thermal efficiency and combustion characteristics of *Jatropha* diesel are very close to petro-diesel. Furthermore, the fact that *Jatropha* biodiesel has a higher cetane number than petro-diesel makes the biodiesel ideal for CI engines with simple or no modifications (Reddy and Ramesh 2006). The ever-increasing demand of *Jatropha* biodiesel in future will imminently require increment of plantations by many folds in an economically profitable way. *Jatropha* plants with improved candidate plus traits, namely economically exploitable seed yield and oil content, desirable biodiesel traits of seed oil, plant architecture, synchronous maturity of fruits, biotic and environmental stress tolerance etc. will be the requisition of foreseeable future. As discussed in this review, some pilot experiments for improvement of major agronomic traits of *Jatropha* have already been successfully carried out through concerted biotechnological interventions. However discovery of new genes with subsequent understanding of *J. curcas* omics for elucidation of mechanism of action of these genes is crucial for further enhancement of *J. curcas* agronomic traits. Fig. 2.4 depicts a simplified delineation of the biotechnological approaches and tools involved in *J. curcas* improvement programs.

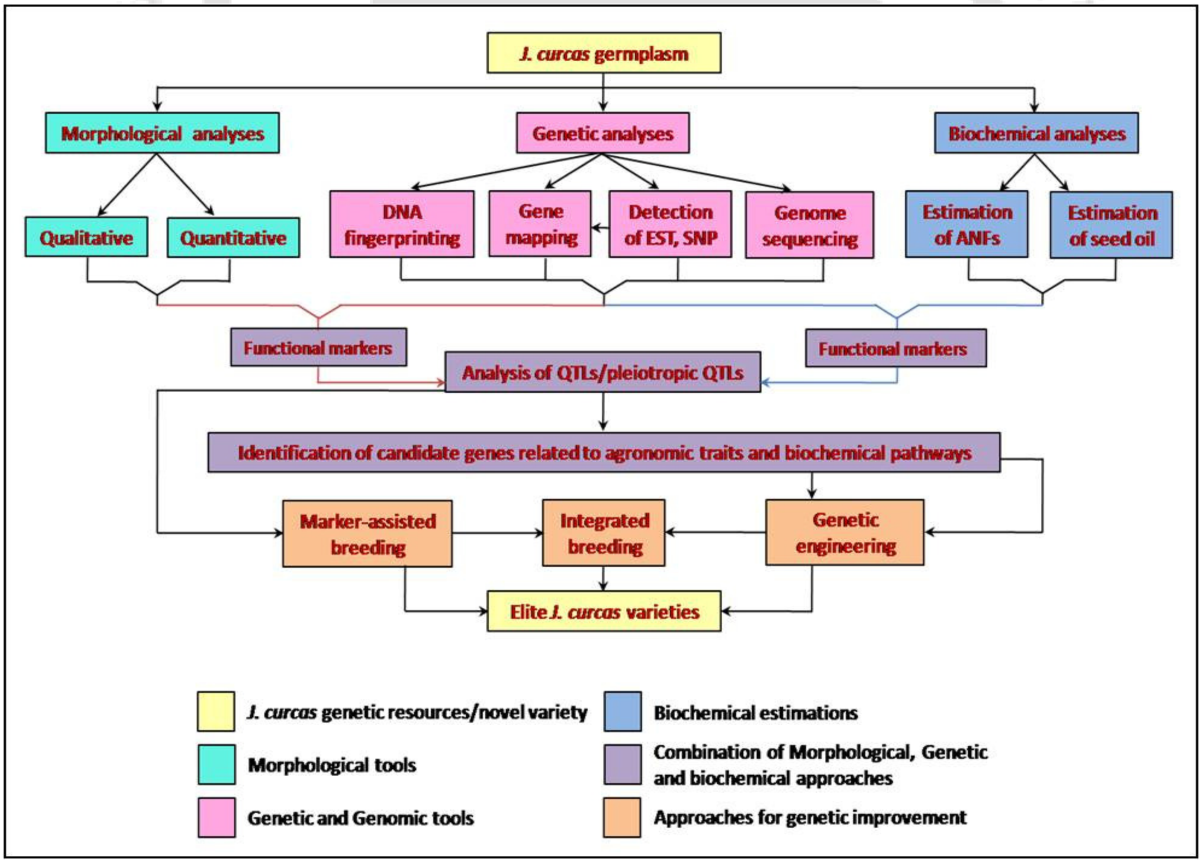


Fig. 2.4 Biotechnological interventions for improvement of *J. curcas*

Breeding and genetic engineering will be the two main potential contributors in *J. curcas* improvement projects. Traditional breeding approaches may help in *J. curcas* improvement but side by side this may lead to the introduction of undesirable characteristics which may have negative effect on the plant health and yield traits. In contrast to conventional methods, molecular marker assisted breeding approaches have adequate potential to circumvent the expression of undesirable features through increasing the specificity of the traits of interest to be transferred. However, one major bottleneck that may limit the implementation of marker-assisted breeding approaches in *J. curcas* is the lack of congruence between molecular markers and phenotypic/biochemical traits of interest hitherto. Correlative studies of morphometric, biochemical and molecular characters will help in development of functional markers for facilitation of marker-assisted breeding in *J. curcas*. Molecular breeding mediated approaches will help in improvement of polygenic and complex quantitative traits in *J. curcas* through MAS mediated introgression of the agronomically favorable traits from diverse *J. curcas* accessions or other closely related *Jatropha* species. However, narrow genetic diversity among *Jatropha* germplasm may lead to unavailability of many traits within the *Jatropha* species. Thus, it is not feasible to attain all improvements in *J. curcas* genome through breeding approaches. Alternative strategies like genetic engineering, on the other hand, will assist in introduction of desired genes from distant plant species or sexually incompatible related species to *Jatropha* genome for further improvement of the plant. Furthermore, it can even help in manipulation of the genes which are already available in *Jatropha* for the improvement of particular traits. Thus, entailment of MAS and genetic engineering approaches in a co-ordinate manner will help in optimization of agronomic performance and commercialization of *J. curcas* as the major feedstock of biofuel.

Complementation of molecular fingerprinting and functional genomics approaches has provided geneticist and plant breeders a whole new series of efficient biotechnological markers (ESTs, SNPs, expression QTLs etc) and advanced tools (tilling etc) for future scheme of *Jatropha* research. Researches on development of *J. curcas* EST databases and transferability of EST-derived markers across related species have also been substantially carried on (Wen et al. 2010;

Eswaran et al. 2012; Chen et al. 2011; SaiSug and Ukoskit 2013). Till date, 46944 EST sequences of *J. curcas* have been made available in NCBI EST database [3]. EST databases are considered as valuable resources of gene discovery. Thus, incorporation of ESTs in *Jatropha* genome mapping projects will help in development of high resolution maps which will subsequently lead to the identification of genes of interest via map based cloning. Furthermore, easy availability of large number of EST contigs and unigenes in *Jatropha* in future can provide valuable foundation for construction of comprehensive and informative cDNA microarray databases for gene expression studies. Most importantly, EST databases can also serve as a rich resource for identification of single nucleotide polymorphism (SNP) in *Jatropha*. In recent times, SNP markers have been recognized as a potential tool for diversity assessment and genome-wide association studies (GWAS) (Ganal et al. 2009). SNP markers have already been efficiently employed for assessment of genetic diversity in *J. curcas* and also for detection of indels in candidate gene sequence in *Jatropha* species (Ricci et al. 2012; Popluechai et al. 2011). Survey of natural SNPs and indels in the functional regions of the genome of the plants like *Jatropha* having narrow genetic background is of paramount importance for gathering clues about adaptive traits which have played significant roles in the evolutionary history of the plant. TILLING (Targeting Induced Local Lesions In Genome), a reverse genetics approach, has been efficaciously used for enlargement of genetic resources in major oilseed plants like *Brassica*, sunflower etc. through detection of induced or naturally occurring SNP variations in oil and fatty acid biosynthesis genes (Wang et al. 2010; Kumar et al. 2013). In an initial attempt to uncover natural variability in candidate genes of *Jatropha*, EcoTILLING, an adaptation of TILLING, was successfully employed for identification of naturally occurring SNPs in genes related to agronomically important traits like stress tolerance, toxin and oil metabolism (Maghuly et al. 2011). In future, TILLING can find application in *Jatropha* breeding programs a cost-effective and high-throughput technique for screening and identification of induced or naturally occurring SNPs having functional consequences in qualitative and quantitative traits of the plant. The sheer abundance of SNPs in plant genome often makes them applicable in molecular breeding studies through the generation of high resolution linkage map. Linkage maps have already been constructed for oleogenic plants like oilseed rape and flax (Qiu et al.

2006; Cloutier et al. 2011). Recently, Wang et al. (2011) used 290 EST-derived SNP markers together with 216 microsatellites for construction of first generation linkage map in *Jatropha*. In a concurrent study, the same research group mapped QTLs and expression QTLs linked with oil traits and oleosin genes respectively in *Jatropha* genome through composite interval mapping (Liu et al. 2011). Availability of *Jatropha* molecular markers and genome sequence will eventually foster development of high density linkage maps with successive localization of QTLs specific to morphometric or biochemical traits in near future. Furthermore, the accessibility of repertoire of genome sequence will probably open new avenues in *Jatropha* improvement ventures with subsequent contribution towards rising demand of biofuels worldwide.

Chapter 3

Morphometric analysis of *J. curcas* populations

3.1 Introduction

Jatropha curcas (Euphorbiaceae) has recently gained worldwide importance as a sustainable source of biodiesel. The biodiesel obtained from *J. curcas* seed oil is an important replacement of petroleum-based diesel fuel (Chitra et al. 2005). *J. curcas* plant is a native of Mesoamerica. However, it has been distributed throughout the arid, semi-arid, tropical and subtropical regions of the world (Augustus et al. 2002). *J. curcas* was introduced to India by Portuguese seafarers during sixteenth century (Sunil et al. 2008). In India, *J. curcas* has acclimatized itself to diverse eco-geographical zones with different edapho-climatic conditions and thus has accumulated lots of variability within the germplasm.

For successful up gradation and commercial exploitation of the biofuel plant, *J. curcas* improvement programs should aim at agronomic traits like low male to female flower ratio, high seed yield with high oil content, abiotic and biotic stress resistance, high natural ramification of branches with greater canopy spread. However, *J. curcas* crop improvement programs are largely dependent on the variability available within the germplasm (Divakara et al. 2010). Thus, assessment of trait based variability in *J. curcas* is prerequisite for screening and selection of agronomically elite genotypes which can later be exploited in breeding programs. Up till now, phenotypic diversity studies in *J. curcas* have mainly focused on assessment of variability in seed traits and seed-oil content (Ginwal et al. 2005; Kaushik et al. 2007; Mazumdar et al. 2012). However, systematic studies involving other morphological and agronomically important traits in *J. curcas* germplasm like plant height, canopy spread, male to female flower ratio, collar length, total seed yield and 100 seed weight for selection of elite planting material is very few (Rao et al. 2008; Srivastava et al. 2011).

The North-East region of India comprises of eight states viz., Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura. Diverse climatic conditions (tropical to temperate), high variation in altitude (50 m to 7000 m) along with high rainfall (800 mm to 4000 mm) has made this agroclimatic zone richest reservoir of plant diversity. In North-East India, *J. curcas* mostly grows in wild conditions. The wild populations from North-

East with a longer history of isolation and evolution harbors significant amount of diversity (Ranade et al. 2008). The diverse populations can contribute towards broadening *J. curcas* genetic resources. Thus, morphometric trait based characterization of *J. curcas* from distinct eco-geographical regions of North-East India has the potential for identification of agronomically elite *J. curcas* plants. However, little research has been conducted on morphological characterization of *J. curcas* germplasm from North-East India (Saikia et al. 2009). The main objective of this study was to assess morphometric diversity of *J. curcas* gerplasm from North-East India along with identification of elite populations.

3.2 Materials and methods

3.2.1 Collection of plant material

Plant collection trips were undertaken during the months of June-August, 2008 for collection of *J. curcas* seeds from wild or unprotected plants. Seeds were collected from six states of North-East India (viz., Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram and Tripura) covering twenty nine distinct eco-geographical regions. *J. curcas* seeds from four other states of India viz., Delhi, Punjab, Gujarat and Orissa, were also included in the study as outgroup (Table 3.1, Fig. 3.1).Plantlets were germinated from the collected seeds and maintained in the shade house of Centre for Energy, IIT Guwahati. Ten different plants from each region were treated as a single population and assigned an accession number (Table 3.1).

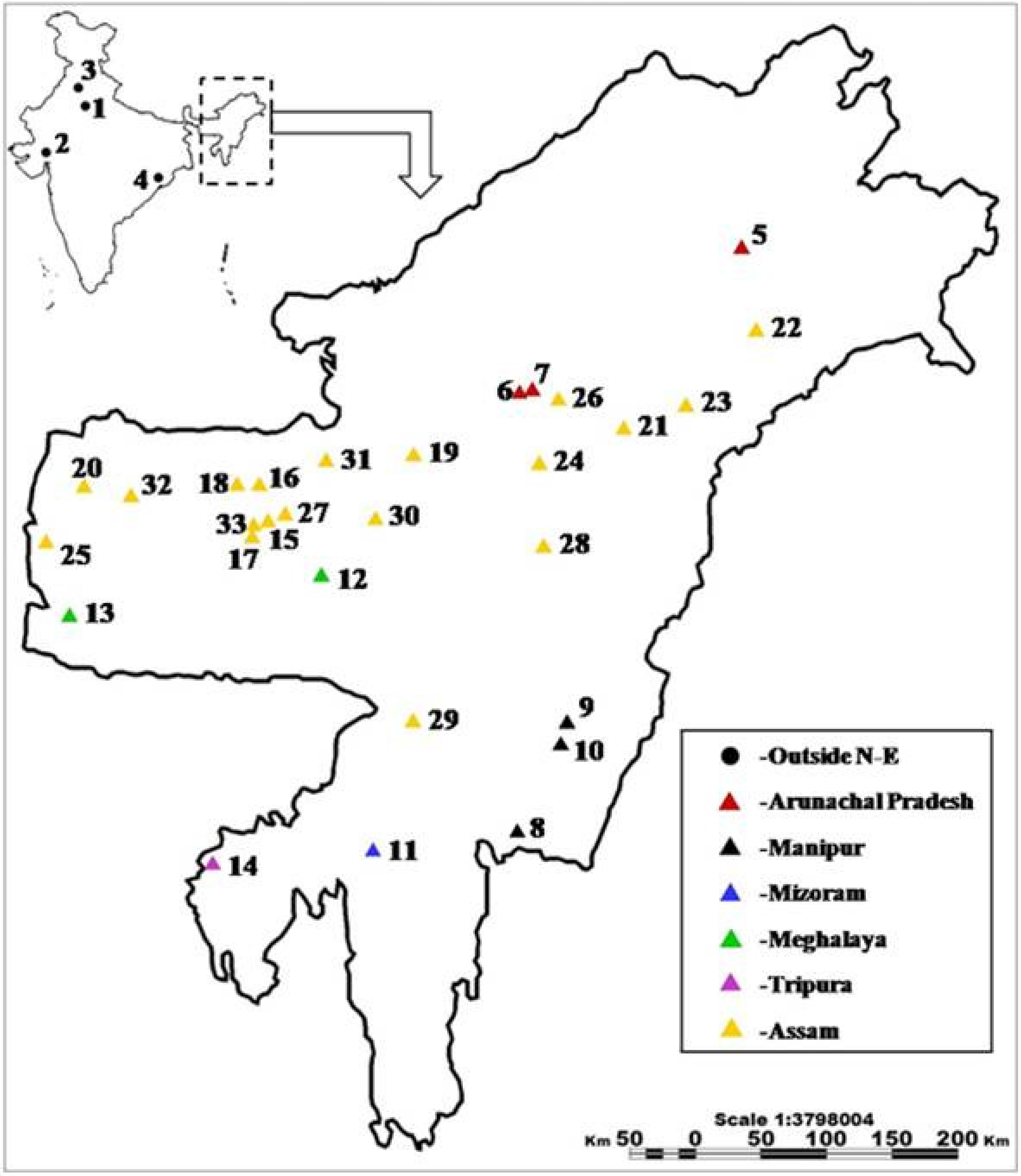


Fig. 3.1 Geographical locations of *J. curcas* populations investigated in this study

Table 3.1 - Geographical locations of *Jatropha curcas* used in diversity analysis

Sl. No.	Population No.	Location of collection	Geographical Location	
			Latitude (°N)	Longitude (°E)
1	IITJC1	Delhi	28·46	77·23
2	IITJC2	Ahmedabad (Gujarat)	23·03	72·58
3	IITJC3	Patiala (Punjab)	30·33	76·40
4	IITJC4	Bhubaneswar (Orissa)	20·15	85·50
5	IITJC5	Pasighat (East Siang, Arunachal Pradesh)	28·07	95·33
6	IITJC6	Itanagar (Papum Pare, Arunachal Pradesh)	27·08	93·40
7	IITJC7	Naharlagoon (Papum Pare, Arunachal Pradesh)	27·10	93·70
8	IITJC8	Hiangtam (Churachandpur, Manipur)	24·05	93·57
9	IITJC9	Imphal (Imphal West,Manipur)	24·48	93·56
10	IITJC10	Wangoi (Imphal West, Manipur)	24·65	93·89
11	IITJC11	Mamit (Mamit, Mizoram)	23·56	92·29
12	IITJC12	Mawhati (Ri Bhoi, Meghayalaya)	25.49	92.50
13	IITJC13	Tura (West Garo Hills, Meghalaya)	25.30	90.16
14	IITJC14	Agartala (West Tripura, Tripura)	23·50	91·23
15	IITJC15	Amingaon (Kamrup, Assam)	26·11	91·40
16	IITJC16	Sitara (Rangia, Assam)	26·44	91·61
17	IITJC17	Sondora (Kamrup, Assam)	26·08	99·56
18	IITJC18	Samota (Nalbari, Assam)	26·44	91·44
19	IITJC19	Tezpur (Sonitpur, Assam)	26·40	92·45
20	IITJC20	Kharigaon (Kokrajhar, Assam)	26·41	90·27
21	IITJC21	Teok (Jorhat, Assam)	26·80	94·39
22	IITJC22	Makum (Tinsukia, Assam)	27·50	95·45
23	IITJC23	Mathurapur (Sibsagar, Assam)	26.98	94.88
24	IITJC24	Numaligarh (Golaghat, Assam)	26·63	93·75
25	IITJC25	Dhubri (Dhubri, Assam)	26·02	89·59
26	IITJC26	Bihpuria (Lakhimpur, Assam)	27·03	93·90
27	IITJC27	Kuruwa (Darrang, Assam)	26·13	91·46
28	IITJC28	Bokajan (Karbi Anglong, Assam)	26·01	93·78
29	IITJC29	Silchar (Cachar, Assam)	24·49	92·48
30	IITJC30	Raha (Nogaon, Assam)	26·23	92·51
31	IITJC31	Jagi Road (Marigaon, Assam)	26·61	92·12
32	IITJC32	Salmala (Bongaigaon, Assam)	26.35	90.63
33	IITJC33	Sualkuchi (Kamrup, Assam)	26.16	91·57

3.2.2 Measurement of plant morphometric characters

The study on phenotypic assessment of morphological descriptors (plant height (PH), collar diameter (CD), canopy spread (CS), floral sex ratios (M:F)) were conducted during flowering time in the third year of plant growing for 33 *J. curcas* populations with 10 plants per population. PH and CS were measured using standard measuring tapes. CD was calculated from girth of the main stem measured 5 cm above the ground. The mature seeds were harvested from the same plants and sun-dried to constant weight. The weight of 100 seeds (100SW) and total seed yield (TSY) from 10 plants per population were estimated. The quantitative data were statistically analyzed and mean value of each morphological trait for individual population, standard error and coefficient of variation was calculated (Table 3.2).

3.2.3 Statistical analysis

Phenotypic inter-relations using the quantitative data were assessed using Manhattan dissimilarity coefficients. Manhattan coefficient was calculated as, $M_{ij} = 1/n \sum_{k=1}^{n} \left| X_{ki} - X_{kj} \right|$, where X_{ki} and X_{kj} are the observed values of two populations i and j with respect to the kth trait and n is the number of morphometric traits considered. The pairwise dissimilarity matrix based on Manhattan coefficient was subjected to cluster analysis using Unweighted Pair Group Method with Arithmetic mean (UPGMA) analysis (Sneath and Sokal 1973). Principal Components Analysis (PCA) was performed to further elucidate phenotypic variability of *J. curcas*. All calculations were done using NTSYS-pc version 2.02 (USA) (Rohlf 1998). Pairwise Pearson correlation coefficients were computed using SigmaPlot 11.0 for determination of the linear relationship among the morphometric traits (Wass 2009). The analysis of variance (ANOVA), broad-sense heritability, phenotypic and genetic variance were calculated for the six selected quantitative traits using the online software PBSTAT 1.2 [4] (Suwarno et al. 2008).

Table 3.2 - Morphological characterization of *Jatropha curcas* populations

Sl. No.	Sample code	Mean PH (cm)	Mean CD (cm)	Mean CS (cm)	Mean M:F ratio	Mean 100SW (g)	Mean TSY (g)
1	IITJC1	185.4±0.95	18.6±0.45	120.4±0.63	24.0:1	54.4±0.81	110.8±0.72
2	IITJC2	168.4±1.94	11.4±0.40	116.1±0.54	16.5:1	68.4±0.96	149.9±1.38
3	IITJC3	180.8±1.10	14.8±0.35	138.6±1.04	22.1:1	72.7±1.00	98.4±0.95
4	IITJC4	143.0±0.94	12.8±0.29	104.5±0.61	16.0:1	76.5±1.03	158.8±1.07
5	IITJC5	162.4±1.37	14.5±0.35	129.6±0.89	14.3:1	78.8±0.94	180.2±1.11
6	IITJC6	280.6±2.24	24.0±0.69	137.4±0.68	13.2:1	96.8±0.91	196.8±1.18
7	IITJC7	268.7±1.90	23.7±0.67	148.4±0.88	14.1:1	123.8±1.01	166.7±1.19
8	IITJC8	269.6±1.45	16.8±0.37	139.7±0.58	13.9:1	88.5±0.84	175.8±1.12
9	IITJC9	240.9±1.43	17.9±0.32	152.2±0.57	14.4:1	116.2±0.96	184.0±1.12
10	IITJC10	277.7±2.16	24.2±0.55	159.7±0.61	12.7:1	95.3±0.47	226.9±1.37
11	IITJC11	263.9±2.72	14.8±0.74	138.2±0.77	13.2:1	62.5±0.84	215.7±1.30
12	IITJC12	194.4±1.54	15.6±0.15	143.3±0.65	13.8:1	67.8±0.51	232.4±1.13
13	IITJC13	178.6±1.65	14.2±0.43	149.2±0.61	14.4:1	71.5±0.63	221.9±1.14
14	IITJC14	177.6±2.01	16.8±0.45	136.7±0.53	14.0:1	68.5±0.32	206.2±1.19
15	IITJC15	191.1±1.54	9.6±0.41	124.7±0.56	10.5:1	85.8±0.64	283.2±1.64
16	IITJC16	298.0±1.30	10.4±0.86	192.5±1.38	13.0:1	83.5±0.61	218.2±1.59
17	IITJC17	153.7±1.50	5.8±0.35	107.3±0.52	19.1:1	89.5±0.47	91.1±1.23
18	IITJC18	198.3±0.87	18.9±0.52	156.2±0.74	13.0:1	66.8±0.75	222.8±1.22

Contd…

Table 3.2 - Morphological characterization of *Jatropha curcas* populations

Sl. No.	Sample code	Mean PH (cm)	Mean CD (cm)	Mean CS (cm)	Mean M:F ratio	Mean 100SW (g)	Mean TSY (g)
19	IITJC19	336.2±1.73	14.4±0.96	229.6±1.46	11.8:1	71.8±0.78	248.2±1.08
20	IITJC20	223.7±1.67	13.6±0.33	177.8±0.54	13.3:1	69.3±0.56	225.1±1.05
21	IITJC21	329.1±2.37	16.8±0.45	237.2±1.62	13.8:1	70.5±0.57	209.1±1.21
22	IITJC22	376.4±2.16	11.8±0.41	191.9±0.55	12.9:1	74.9±0.64	231.3±1.76
23	IITJC23	287.2±1.43	8.6±0.24	149.2±0.61	25.4:1	74.7±0.51	118.8±0.81
24	IITJC24	385.7±2.03	11.8±0.59	239.9±1.47	12.5:1	71.0±0.33	216.5±1.49
25	IITJC25	282.7±1.48	15.8±0.41	147.2±0.39	16.4:1	87.5±0.59	169.2±1.32
26	IITJC26	159.8±1.53	11.4±0.89	134.8±0.48	14.0:1	78.4±0.46	207.8±1.28
27	IITJC27	310.8±1.82	8.8±0.39	145.3±0.56	12.4:1	84.4±0.71	223.9±1.99
28	IITJC28	192.3±1.19	30.5±0.95	154.8±0.47	13.7:1	108.4±1.05	200.4±1.30
29	IITJC29	182.8±0.99	15.4±0.47	141.5±0.82	16.0:1	65.3±0.47	155.6±1.00
30	IITJC30	289.8±1.28	9.5±0.49	163.4±0.90	12.5:1	79.5±0.92	235.9±1.92
31	IITJC31	258.7±1.53	7.2±0.28	132.5±0.65	11.5:1	81.1±0.69	241.6±1.60
32	IITJC32	145.9±1.50	14.6±0.38	133.2±1.24	14.1:1	74.3±0.36	198.3±1.08
33	IITJC33	255.1±1.61	9.8±0.62	149.8±0.61	19.5:1	72.5±0.92	172.2±1.73
	Mean	237.9	14.7	152.2	14.9	79.7	193.7
	CV (%)	2.13	11.07	1.67	23.19	2.75	2.05

3.3 Results and discussion

3.3.1. Determination of mean, minimum and maximum values and coefficient of variations

The perusal of all six quantitative traits exhibited considerable morphological variability in 33 *J. curcas* populations under investigation ($p < 0.01$) (Table 3.2). The mean data on morphometric parameters showed a broad range of variation in plant height (PH) (143 cm - 385.7 cm). Significant variations were observed in collar diameter (CD) (8.61 cm - 30.46 cm), seed weight per 100 seeds (100SW) (54.4 g-123.8 g) and total seed yield (TSY) (91.1 g - 283.8 g). Canopy spread (CS) appeared to be the least variable trait (104.5 cm - 239.9cm) with % coefficient of variation (%CV) of 1.67. The highest variation (%CV=23.19) was observed in male to female flower (M:F) ratio (10.5 - 25.4). The population, IITJC24, had highest PH (385.7 cm) and CS (239.9 cm) with respect to all other populations. On the other hand, IITJC15 had maximum TSY (283.2 g) and minimum M:F ratio (10.5:1). IITJC28 had maximum CD (30.5 cm). The population IITJC7 from Arunachal Pradesh recorded highest 100SW (123.8 g).Thus, three populations from Assam (IITJC15, IITJC24 and IITJC28) and one population from Arunachal Pradesh (IITJC7) emerged as superior on the basis of morphometric trait values.

3.3.2 Correlation coefficients

The Pearson correlation between growth attributes and seed characters of *J. curcas* was estimated. The correlation coefficients revealed a positive correlation between the growth traits like PH, CS and CD with TSY (Table 3.3). However, M:F ratio was negatively correlated with all other morphological traits. Interestingly, existence of a highly significant negative association was observed between M:F ratio and TSY. This allows us to hypothesize that a direct positive correlation exists between number of female flowers and TSY. This is in good accordance with Rao et al. (2008) who found a positive relationship of plant height and female to male flower ratio with seed yield. The fact that IITJC3 and IITJC17, the low seed-yielding populations, had below average value for most of the other morphological traits was in corroboration with the correlation analysis data which showed a positive association of all traits (with the exception of M:F ratio) with TSY. Thus correlation analysis allowed direct assessment of positive and negative contribution of other quantitative traits on TSY. From these observations it was inferred that morphometric traits like PH, CS and CD can be used as initial screening indices for selection of higher-yielding *J. curcas* plants at an early stage of growth, i.e., before completion of gestation period. Thus, it was suggested that during *J. curcas* improvement programs, increasing total number of female flowers or producing a more extensive canopy will provide better opportunities for increasing total seed yield of the plant.

Table 3.3 - Pearson correlation coefficients for morphometric traits of *J. curcas* populations

	CD	CS	M:F ratio	100SW	TSY
PH	-0.05	0.77^{**}	-0.26	0.12	0.35^{*}
CD		0.05	-0.12	0.42^{**}	0.01
CS			-0.33	-0.08	0.42^{**}
M:F ratio				-0.24	-0.87^{**}
100SW					0.01

Significance level: * = p≤0.05, ** = p ≤ 0.01

3.3.3 Cluster and Principal Component Analyses

The UPGMA dendrogram based on Manhattan dissimilarity matrix separated the 33 populations into three major clusters I, II and III with 16, 13 and 14 populations respectively. Cluster I and Cluster II again formed four (IA, IB, IC and ID) and three (IIA, IIB and IIC) distinct sub-clusters respectively (Fig. 3.2). The grouping of 33 *J. curcas* populations in eight sub-clusters has been shown in Table 3.4. The outside North-East populations dispersed themselves into sub-clusters IA and IB along with populations from Assam and Arunachal Pradesh. The sub-clusters IC, IIA and IIB consisted of mixed populations from North-East. On contrary, sub-clusters ID, IIC and cluster III were specific to populations from Assam.

Table 3.4 - Grouping of *Jatropha curcas* populations as depicted by cluster analysis

Clusters	Sub-Clusters	No. of populations	Population codes
I	IA	3	IITJC1, IITJC3, IITJC17
	IB	6	IITJC2, IITJC4, IITJC5, IITJC26, IITJC29, IITJC32
	IC	6	IITJC12, IITJC13, IITJC14, IITJC18, IITJC20, IITJC28
	ID	1	IITJC15
II	IIA	6	IITJC6, IITJC7, IITJC8, IITJC9, IITJC25, IITJC33
	IIB	6	IITJC10, IITJC11, IITJC16, IITJC27, IITJC30, IITJC31
	IIC	1	IITJC23
III	III	4	IITJC19, IITJC21, IITJC22, IITJC24

The sub-cluster wise mean values of the quantitative morphological traits were also estimated (Table 3.5). The highest value of mean plant height (356.86 cm) and mean canopy spread (224.7 cm) were observed in cluster III. Alongside, cluster III also showed high values for 100SW and low M:F ratio. Sub-cluster IC comprising of populations from Meghalaya, Tripura and Assam recorded the highest CD (18.3 cm). Sub-cluster IIA consisting of two populations each from Arunachal Pradesh, Manipur and Assam recorded the maximum 100SW (97.6 g). Highest value of TSY and the lowest M:F ratio was observed in sub-cluster ID containing a single population, IITJC15. Thus, cluster III and sub-cluster ID recorded high mean value for majority of the agronomic traits. The populations from cluster III (IITJC19, IITJC21, IITJC22 and IITJC24) and sub-cluster ID (IITJC15) were found promising for the future tree improvement programs.

Table 3.5 - Mean values of morphological traits in *Jatropha curcas* for eight sub-clusters

Clusters	PH (cm)	CD (cm)	CS (cm)	M:F ratio	100SW (g)	TSY (g)
IA	173.3	13.1	122.1	21.7:1	72.2	100.1
IB	160.4	13.4	126.6	15.2:1	73.6	175.1
IC	194.2	18.3	153	13.7:1	75.4	218.1
ID	191.1	9.6	124.7	10.5:1	85.8	283.2
IIA	266.3	18	145.8	15.3:1	97.6	177.5
IIB	283.2	12.5	155.3	12.6:1	81.1	227
IIC	287.2	8.6	149.2	25.4:1	74.7	118.8
III	356.86	13.7	224.7	12.8:1	72.1	226.3

In order to gain a better comprehension of the relationship among the *Jatropha* populations, Principal Component Analysis (PCA) was done by considering all the six morphometric traits concurrently (Fig. 3.3). PCA showed separation of the 33 populations into three discrete groups (I, II and III). Groups I and II was again sub-divided into four and three sub-groups respectively. The overall grouping pattern of the populations in PCA was in accordance with the major clades of the UPGMA dendrogram. It has been previously reported that crossing of populations from the clusters, which are having maximum inter-cluster distance and high mean value of agronomic traits, would result in production of more divergent trees (Shabanimofrad et al. 2013; Srivastava et al. 2011; Kaushik et al. 2007). Both the analyses results delineated that the four populations in cluster III, IITJC19, IITJC21, IITJC22 and IITJC24, have maintained maximum inter-cluster distance from other *J. curcas* populations. Thus, it can be envisaged that selection of parents from these four populations during breeding programs would lead to the development of *J. curcas* plants with greater genetic heterogeneity.

When the grouping pattern of outside North-East populations were compared with North-East populations, it was observed that in both cluster analysis and principal component analysis, *J. curcas* populations from outside North-East have nested together with populations from North-East India. Thus, from morphological-character-derived cluster analysis and principal component analysis it was inferred that for all morphometric traits, association among populations was independent of their geographic origin. The inefficacy of dendrogram and PCA plot to reveal a clear relationship between diversity pattern and geographical origin led to the speculation that morphometric traits of *J. curcas* are relatively uncorrelated with geographic distribution.

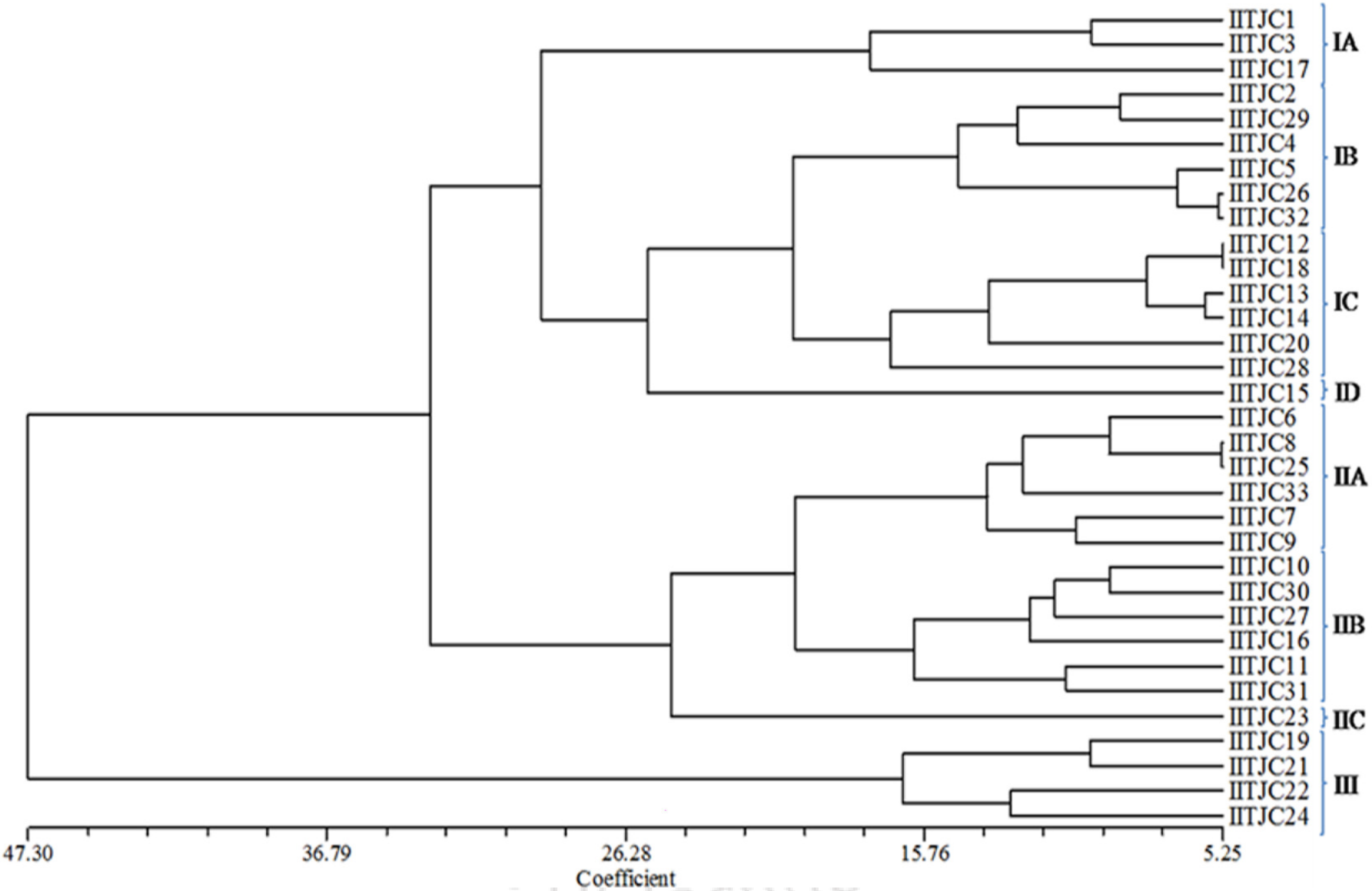


Fig. 3.2 Morphometric relationship among 33 *J. curcas* populations based on UPGMA based cluster analysis

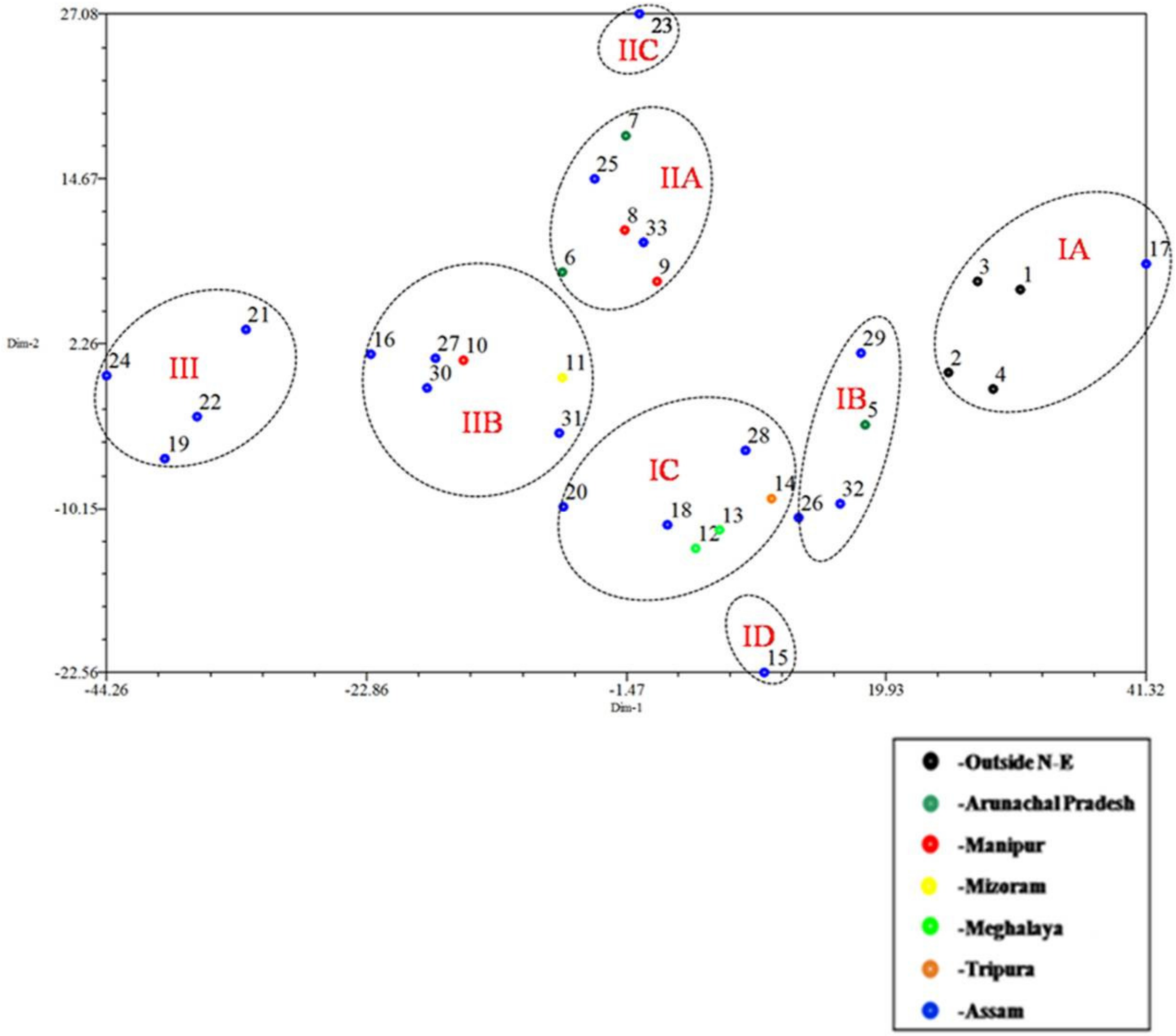


Fig. 3.3 Morphometric relationship among 33 *J. curcas* populations based on Principle component analysis

3.3.4 Analysis of variance & estimation of variance components

An ANOVA for morphometric traits reflected highly significant differences between the *J. curcas* accessions under study at p≤0.01 (Table 3.6). ANOVA among *J. curcas* accessions for different morphometric characters have previously been reported (Shabanimofrad et al. 2013; Sunil et al. 2012b).

Table 3.6 - Analysis of variance for morphometric traits in 33 *J. curcas* populations

		Mean Sum of Squares					
Source	**df**	**PH**	**CD**	**CS**	**M:F ratio**	**100SW**	**TSY**
Replicates	9	89.6^{**}	8.3^{**}	19.3^{**}	100.2^{**}	26.9^{**}	63.3^{**}
Populations	32	46001.2^{**}	283.9^{**}	$11,000.60^{**}$	119.4^{**}	2261.6^{**}	19982.4^{**}
Error	288	25.6	2.6	6.5	12	4.8	15.8

Significance level: $^{**} = p < 0.01$

In selection and breeding experiments, knowledge of heritability and phenotypic trait under selection is essential for predicting the selection response and improving the agronomic trait through selection (Visscher et al. 2008; Robinson et al. 1949). Thus, broad sense heritability (h^2_{bs}) and variance components (phenotypic variance, V_P and genotypic variance, V_G) were estimated for all six agronomic traits in *J. curcas* (Table 3.7). The estimates of V_P as compared to V_G were higher for all traits. Broad-sense heritability is defined as the ratio of total genetic variation to total phenotypic variation (Brown et al. 2012). Thus, the high magnitude of h^2_{bs} (65%) for all morphological traits depicted the dominance of heritable variation in *J. curcas*. Comparatively high % of h^2_{bs} (> 75%) was observed for seed yield related traits like M:F ratio, 100SW and TSY respectively. However, on the other hand, low h^2_{bs} (< 72%) was detected for plant growth related traits like PH, CD and CS. High estimates of heritability for M:F ratio (88.07%), 100SW (82.19%) and TSY (75%.01) also envisaged that environment has relatively less influence on the seed yield related traits. Therefore, from the present study, it can be inferred that selection of elite plants on the basis of M:F ratio, 100SW and TSY (h^2_{bs} > 75%) in tree improvement programs is likely to be more successful.

Table 3.7 - Estimation of variance components and broad sense heritability

Variable	**V_G**	**V_P**	**h^2_{bs} (%)**
PH	1.9397	2.7166	71.40
CD	0.1716	0.2517	68.2
CS	0.3882	0.5847	66.39
M:F ratio	2.6735	3.0356	88.07
100SW	0.6703	0.8155	82.19
TSY	1.4394	1.919	75.01

3.4 Conclusion

The present study reflected high level of morphometric variation among 33 *J. curcas* populations. The populations IITJC7, IITJC15, IITJC24 and IITJC28 were identified as superior on the basis of morphometric trait values. Cluster and principal component analysis demarcated IITJC15, IITJC19, IITJC21, IITJC22 and IITJC24 as promising and diverse populations. The seven populations from North-East India (IITJC7, IITJC15, IITJC19, IITJC21, IITJC22, IITJC24 and IITJC28) identified in this investigation on the basis of morphometric trait values and cluster analysis results can be recommended as potential starting materials in tree breeding programs for the development of genetically diverse *J. curcas* genotypes with desirable agronomic traits. All seven promising populations were advanced for further studies related to estimation of *J. curcas* seed phorbol ester content.

Chapter 4

Molecular marker based genetic diversity studies of *J.curcas*

4.1 Introduction

The global energy crisis due to depletion of fossil fuel and growing perils of global warming have prompted renewed interest in alternative renewable energy source for sustainable development of economy. The plant seed-derived biofuel offers unique opportunities for environment friendly sustainable energy. *Jatropha curcas* has emerged as an important biodiesel crop worldwide. *J. curcas* seed oil possesses the desired physiochemical characteristics for direct blending with petro-diesel or use as biodiesel after transesterification (Jones and Miller 1992; Sahoo and Das 2009). Being a perennial non-edible plant, *J. curcas* offers unique opportunities of high seed biomass production for biodiesel, a feedstock that does not compete with other oil seed crops. Hardiness, rapid growth, easy propagation, drought endurance, short gestation period, optimum plant size together with wide adaptability to adverse agro-climatic conditions makes this species ideal for cultivation on wastelands (Reddy and Pamidimarri 2010). Large scale cultivation remains the single most important determinant for the success of *J. curcas* as a bio-fuel crop. However, limited information on the genetics of this species, inconsistent seed yield, narrow genetic variability, and susceptibility to insects and diseases pose major constraints in successful its commercial scale exploitation as a bio-fuel crop (Prabakaran and Sujatha 1999).

Adaptation of *J. curcas* to a wide range of edaphic and ecological conditions at diverse geographical locations signifies the existence of a considerable amount of genetic diversity to be exploited for potential realization (Ranade et al. 2008). DNA polymorphisms detected during molecular marker based characterization can be efficiently used for selection of highly divergent genotypes as well as marker-assisted culling of redundancy in *J. curcas* germplasm. Polymerase chain reaction (PCR) based molecular-typing approaches including Random Amplified Polymorphic DNA (RAPD) (Williams et al. 1990) and Inter Simple Sequence Repeats (ISSR) (Zietkiewicz et al. 1994) have been efficiently employed to analyze inter- and intra-specific genetic diversity in *J. curcas* (Basha and Sujatha 2007; Gupta et al. 2008; Pamidimarri et al. 2008; Pamidimarri et al. 2010). In recent years many promising gene-targeted marker techniques have emerged as powerful tool to identify genetic variability. Toxicity related curcin gene-specific markers have been previously used for assessing diversity of *J. curcas* populations (Basha et al. 2009; Ricci et al. 2012). Thus, coupled with random DNA marker systems, gene-targeted markers such as curcin gene-specific marker can supplement information on molecular marker based gene diversity studies in *J. curcas*.

North-East India, being a part of both trans-Himalayan mountainous zone and Indo-Burma biodiversity hotspot, represents the richest plant diversity reservoir in India (Myers et al. 2000; Mao et al. 2009). *J. curcas* is now well-naturalized in this unique biogeographic region (Mazumdar et al. 2012). Kumar et al. (2011; 2013) recently reported the genetic diversity analysis of *J. curcas* germplasm covering only two states (Assam and Meghalaya) of North-East India. However, *J. curcas* genetic diversity from major part of North-East has not been assessed hitherto. Therefore, the present study was undertaken to estimate the genetic diversity for identification of elite populations of *J. curcas* from North-East India using random markers (RAPD and ISSR) and gene-targeted marker (curcin).

4.2 Materials and methods

4.2.1 Plant material

Twenty nine *J. curcas* populations from North-East India and four populations from outside North-East India studied in Chapter 3 (Table 3.1) were used for studying of molecular diversity.

4.2.2 DNA extraction

Approximately 0.5 g of young, tender leaves from ten individual plants from each population was bulked for DNA extraction. The total genomic DNA was extracted from pulverized leaf samples of 33 populations of *J. curcas* following the standard CTAB method with minor modifications (Doyle and Doyle 1990). The quality and concentration of the DNA samples were analyzed by spectrophotometric analysis and agarose gel electrophoresis to confirm DNA integrity and RNA contamination. The extracted DNA was diluted to final concentrations of 100 ng/ µl for RAPD analysis and 50 ng/ µl for ISSR and curcin specific analyses respectively.

4.2.3 PCR amplification

4.2.3.1 Primer survey and selection

A total of forty RAPD (Operon technologies, USA) and twenty ISSR (UBC primer set No. 9, University of British Columbia, Canada) primers were screened initially with five test populations for polymorphism and reproducibility. Eighteen RAPD and thirteen ISSR primers (Table 4.1) were selected for subsequent screening based on their reproducible and polymorphic marker profiles.

4.2.3.2 RAPD analysis

The RAPD assay was carried out in 25 µl reaction volume containing 100 ng genomic DNA, 100 µM of dNTP mix, 1 µM of random primer and 1.0 U of Taq DNA polymerase (Bangalore Genei, India). Amplification was performed in thermal cycler (Applied Biosystems 2720, USA).The standardized amplification for RAPD was: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min; primer annealing at 34° C for 1 min; primer extension at 72°C for 2 min and final primer extension at 72°C for 10 min. The PCR products were size-separated on 1.5% agarose gel in 1X TBE buffer by electrophoresis at 100 V for 3 h and visualized in gel documentation system (Bio-Rad Laboratories, USA).

4.2.3.3 ISSR analysis

For ISSR analysis PCR amplification was carried out using 50 ng of template DNA, 100 µM of dNTP mix, l µM of primer and 1.0 U of Taq DNA polymerase (Bangalore Genei, India) in a final volume of 25 µl. PCR amplification was programmed with initial denaturation at 94°C for 3 min, followed by 40 cycles for 1 min at 94°C, 1 min at primer annealing temperature and 2 min at 72°C and final primer extension at 72°C for 10 min. The amplicons were electrophoresed at 100V for 3 h in 1.7% agarose gel and visualized for data registration.

4.2.3.4 Curcin-specific primer

The curcin region was amplified by the primer combinations Curcin-P2 forward (5′-CGTGTCATATTTCTCGTTTG-3′) and reverse primers (5′-GAGATAGAATGGTCACAAGT-3′) (Basha et al. 2009). PCR amplifications were carried out in a final volume of 25 µl, containing 50 ng of template DNA, 100 µM dNTP mix, 0.5 µM each of forward and reverse primers, and 1.0 U of Taq DNA polymerase. PCR amplifications were performed with an initial denaturation at 94°C for 3 min followed by 35 cycles of 1 min at 94°C, 45 s at 54°C, 2 min elongation at 72°C with a final extension at 72°C for 10 min. The amplified products were resolved by electrophoresis on 2.5% agarose gel.

4.2.4 Scoring and data analysis

The RAPD, ISSR and curcin gene-specific amplicons were analyzed as alleles assuming presence of Hardy-Weinberg equilibrium and segregation of all loci in a dominant Mendelian fashion. Only distinct and consistent amplicons were scored in a binary fashion attributing 1 as presence and 0 as absence of amplicons. Each amplicon was considered as an independent character and rectangular matrices of binary data were generated for RAPD, ISSR, combined RAPD and ISSR, and curcin markers.

The presence and absence data matrices for RAPD, ISSR, combined RAPD and ISSR were then used for the computation of following population genetic parameters using POPGENE version 1.32 (Francis and Yang 2000) : 1) the number and percentage of polymorphic bands; 2) the average number of alleles per loci (Na); 3) the effective number of alleles per loci (Ne); 4) Nei’s gene diversity (H) ($\mathrm{H} = \mathrm{H}_\mathrm{T} - \mathrm{H}_\mathrm{S}$, where H_T = total gene diversity and H_S= gene diversity within the population) (Nei 1973) and Shannon’s information index ($\mathbf{I}$) ($\mathrm{I} = -\mathrm{P}_\mathrm{i}/\log_2 \mathrm{P}_\mathrm{i}$, where P_i= gene frequency in locus i) (Lewonton 1972). For POPGENE-based analysis, the 33 populations were divided into two groups, North-East and outside North-East regions based on their geographic location.

The analysis of molecular variance (AMOVA) was performed with RAPD, ISSR, combined RAPD and ISSR datas for estimation of the variance components at two hierarchical levels, and to partition the total genetic variation between two distinct regions (North-East and outside North-East) and also among the 7 groups (Arunachal Pradesh, Manipur, Mizoram, Meghalaya, Tripura, Assam and outside North-East) using AMOVA version 1.55 (Excoffier et al. 1992). AMOVA-PREP 1.01 was used to generate input files required for the AMOVA analysis (Miller 1998). The statistical significance of variance components were assessed non-parametrically with 1000 randomized permutations.

Pairwise matrix of similarity between genotypes was generated for RAPD, ISSR, combined RAPD and ISSR markers by Jaccard's coefficient of similarity, $J_{ij} = a/(a + \mathrm{b} + c)$ where J_{ij} is the measure of genetic similarity between populations i and j, a is the number of polymorphic bands present in both populations, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i(Jaccard 1908). Dendrograms were constructed on the basis of Jaccard's coefficient of similarity for all the marker systems by using UPGMA with the SAHN module of NTSYS-pc (Rohlf 1998). FreeTree program (ver. 0.9.1.5) was used for validation of cluster analysis with 1000 bootstraps (Pavlicek 1999).

Principal Component Analysis (PCA) was performed to elucidate genetic diversity structuration of studied populations using the EIGEN and MXPLOT modules of NTSYS-pc. Mantel test was performed to test the correspondence between the similarity matrices based on RAPD, ISSR and combined RAPD + ISSR datasets and validated by 10,000 permutations of bootstrapping (Mantel 1967).

4.3 Results and discussion

4.3.1 RAPD analysis

The details of the RAPD primers used in the present study have been presented in Table 4.1. Approximately 192 amplicons (0.2 – 3.7 kb) were scored from RAPD analysis with 18 primers (Table 4.3). This indicated an average of 10.67 amplicons per primer. Around 130 amplicons proved to be polymorphic corresponding to 67.71% of the total amplicons. The primer OPK-01 generated the highest number of polymorphic bands with 90% polymorphism (Table 4.1). The maximum Jaccard's similarity coefficients value (J=0.927) was estimated between IITJC5 and IITJC6 from Arunachal Pradesh while the least similarity (J=0.617) was recorded between two geographically distant populations, IITJC1 from Delhi and IITJC13 from Meghalaya. The banding patterns of RAPD primers OPG-02 and OPS-09 is shown in Fig. 4.1A and Fig. 4.1B respectively.

4.3.2 ISSR analysis

The details of the ISSR primers used in the present study are presented in Table 4.2. In total, 124 amplicons were scored in the size range of 0.2 – 3.0 kb (Table 4.3). The average number of amplicons per primer was 9.54. Of the 124 amplicons, 82 amplicons proved to be polymorphic resulting in a polymorphic frequency of 66.13%. UBC-841 proved to be the most efficient with 100% polymorphism (Table 4.2). The ISSR similarity matrix on the basis of Jaccard's coefficient revealed a moderate level of genetic diversity within the *J. curcas* populations. IITJC16 and IITJC29 from Assam showed the maximum genetic similarity (J=0.893). The minimum genetic similarity (J=0.626) was observed between populations IITJC17 and IITJC24 from Assam. The ISSR fingerprinting patterns of primers UBC-840 and UBC-841 is presented in Fig. 4.1C and Fig. 4.1D respectively.

4.3.3 Combined RAPD and ISSR analysis

Among the two assays, the RAPD analysis explores the entire *J. curcas* genome for detection of polymorphism while the ISSR analysis reveals information about diversity of *J. curcas* populations in microsatellite-rich regions of the genome. Therefore, a combined data analysis of RAPD and ISSR assays is expected to facilitate a comprehensive and precise analysis of genetic diversity among the *J. curcas* populations. A combined analysis involving a total of 31 primers (RAPD+ISSR) resulted in 316 amplicons, of which 212 were polymorphic with an average polymorphic frequency of 67.09% (Table 4.3). The estimated genetic similarity (J=0.889) was found highest between IITJC5 and IITJC6 while the lowest (J=0.623) between IITJC1 and IITJC13.

Table 4.1 Details of RAPD primers used in the present study and the extent of polymorphism

Sl. No.	Primer's name	Primer's sequence (5'-3')	Total no. of bands amplified	No. of polymorphic bands	Percentage polymorphism	Size range of amplified products (kb)
1	OPA-07	GAAACGGGTG	7	6	85.71	0.3 - 3.0
2	OPG-02	GGCACTGAGG	8	7	87.5	0.4 - 2.90
3	OPK-01	CATTCGAGCC	10	9	90.0	0.5 - 1.80
4	OPK-02	GTCTCCGCAA	8	5	62.5	0.25 - 1.0
5	OPK-03	CCAGCTTAGG	11	6	54.55	0.3 - 2.0
6	OPK-04	CCGCCCAAAC	8	5	62.5	0.5 - 2.2
7	OPK-07	AGCGAGCAAG	13	9	69.23	0.5 - 2.7
8	OPK-09	CCCTACCGAC	10	8	80.0	0.45 - 1.9
9	OPK-11	AATGCCCCAG	11	6	54.55	0.5 - 2.0
10	OPK-16	GAGCGTCGAA	8	7	87.5	0.37 - 3.7
11	OPK-20	GTGTCGCGAG	16	10	62.5	0.2 - 1.9
12	OPL-01	GGCATGACCT	5	3	60.0	0.35 - 1.8
13	OPL-03	CCAGCAGCTT	15	11	73.33	0.2 - 2.1
14	OPL-04	GACTGCACAC	16	11	68.75	0.35 - 2.2
15	OPQ-13	GGAGTGGACA	11	5	45.45	0.4 - 1.8
16	OPQ-16	AGTGCAGCCA	10	4	40.0	0.5 - 1.8
17	OPS-09	TCCTGGTCCC	13	11	84.62	0.35 - 3.5
18	OPS-19	GAGTCAGCAG	12	7	58.33	0.2 - 1.9

Table 4.2 Details of ISSR primers used in the present study and the extent of polymorphism

Sl. No.	Primer's name	Primer's sequence (5'-3')	Total no. of bands amplified	No. of polymorphic bands	Percentage polymorphism	Size range of amplified products (kb)
1	I1	(GA)$_9$C	10	6	60.0	0.2 - 0.8
2	UBC-807	(AG)$_8$T	12	8	66.67	0.3 - 1.5
3	UBC-810	(CA)$_8$T	10	8	80.0	0.2 - 1.8
4	UBC-812	(GA)$_8$A	14	9	64.29	0.5 - 3.0
5	UBC-815	(CT)$_8$G	8	6	75.0	0.2 - 2.5
6	UBC-818	(CA)8G	10	6	60.0	0.2 - 2.3
7	UBC-840	(GA)$_8$YT	9	4	44.44	0.55 - 1.8
8	UBC-841	(GA)$_8$YC	6	6	100.0	0.20 - 1.8
9	UBC-844	(CT)$_8$RC	7	5	71.43	0.25 - 2.0
10	UBC-884	HBH(AG)$_7$	8	5	62.5	0.30 - 1.2
11	UBC-895	AGAGTTGGTAGCTCTTGATC	5	3	60.0	0.55 - 2.7
12	UBC-899	CATGGTGTTGGTCATTGTTCCA	12	8	66.67	0.10 - 2.7
13	UBC-900	ACTTCCCCACAGGTTAACACA	13	8	61.54	0.40 - 2.0

Table 4.3 Comparison of RAPD and ISSR markers

Sl. No.	Marker	No. of primer used	Total bands amplified	Average bands/ primer	Total no. of polymorphic bands	Average no. of polymorphic bands/ primer	Average % polymorphism	Distance range (Jaccard's coefficient)	Average distance
1	**RAPD**	18	192	10.67	130	7.22	67.71	0.617 - 0.927	0.772
2	**ISSR**	13	124	9.54	82	6.31	66.13	0.577 - 0.895	0.736
3	**RAPD+ISSR**	31	316	10.19	212	6.84	67.09	0.70 - 0.89	0.80

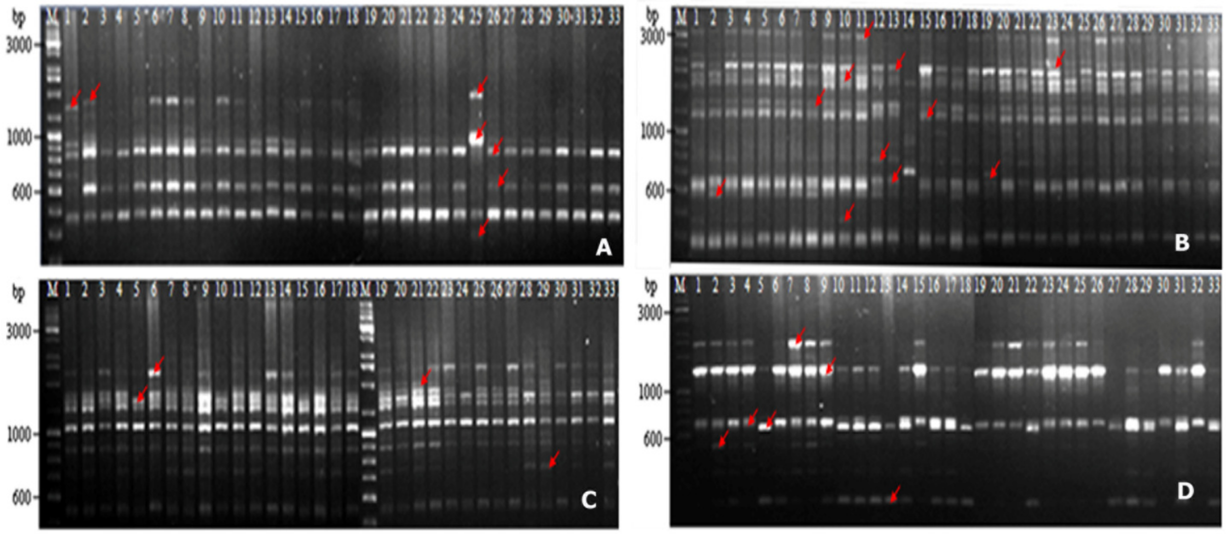


Fig.4.1 PCR amplification profile of *Jatropha curcas* populations using **(A)** RAPD primer **OPG-02**, **(B)** RAPD primer **OPS-09**, **(C)** ISSR primer **UBC-840** and **(D)** ISSR primer **UBC-841**

4.3.4 Curcin-specific marker analysis

The curcin-P2 primer amplified 2 specific fragments in all 33 *J. curcas* populations (Fig. 4.2). Similar monomorphic banding pattern was also reported from Brazil, Mozambique and Senegal in *J. curcas* populations (Ricci et al. 2012). However, our result was in discordance with other study (Basha et al. 2009). The similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between *J. curcas* populations was 1, indicating 100% similarity indicating that curcin-P2 primer was non-informative and the monomorphic datas were not included in genetic diversity and AMOVA analyses.

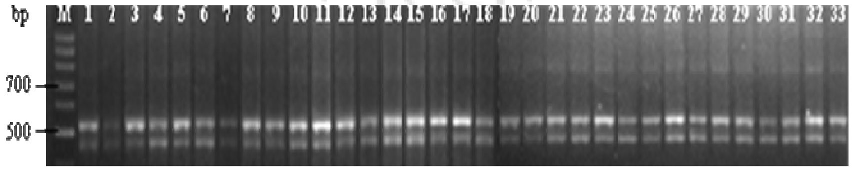


Fig. 4.2 PCR amplification profile of *Jatropha curcas* populations using Curcin-P2 primer

4.3.5 Genetic diversity analysis

The comparative data of genetic diversity of *J. curcas* populations from North-East and outside North-East regions is summarized in Table 4.4. The populations from North-East region revealed relatively high value of Nei's gene diversity (H) and Shannon's Information index (I) for RAPD (H = 0.182, I = 0.2798), ISSR (H = 0.1749, I = 0.27) and combined RAPD and ISSR (H = 0.1716, I = 0.2701) when compared with the outside North-East populations. The results indicated that *J. curcas* populations harboring comparatively higher genetic variations remained well conserved in the North-East Himalayan region of India. Moreover, in this study, RAPD, ISSR and combined RAPD and ISSR datasets were found to be equally efficient and informative based on observed number of alleles (Na), effective number of alleles (Ne), H and I values.

Table 4.4 - Comparison of genetic diversity estimates in *Jatropha curcas*

Regions	Na ± SD	Ne ± SD	H ± SD	I ± SD
RAPD				
Outside N-E	1.2292 ± 0.4214	1.1684 ± 0.3353	0.0935 ± 0.1788	0.1359 ± 0.2559
N-E	1.6302 ± 0.4840	1.3043± 0.3583	0.1820 ± 0.1902	0.2798 ± 0.2695
Total	1.6771 ± 0.4688	1.3097 ± 0.3545	0.1865 ± 0.1880	0.2884 ± 0.2649
ISSR				
Outside N-E	1.1452 ± 0.3537	1.1141 ± 0.2944	0.0619 ± 0.1553	0.0891 ± 0.2208
N-E	1.6210 ± 0.4871	1.2921 ± 0.3560	0.1749 ± 0.1893	0.2700 ± 0.2675
Total	1.6613 ± 0.4752	1.2831 ± 0.3450	0.1722 ± 0.1839	0.2692 ± 0.2593
RAPD+ISSR				
Outside N-E	1.1962±0.3978	1.1456±0.3071	0.0823±0.1689	0.1194±0.2437
N-E	1.6266±0.4845	1.2722±0.3168	0.1716±0.1735	0.2701±0.2509
Total	1.6709±0.4706	1.2672±0.3056	0.1715±0.1670	0.2736±0.2408

H = Nei's (1973) gene diversity; I = Shannon's Information index (Lewontin 1972); Na = Observed number of alleles; Ne = Effective number of alleles (Kimura and Crow 1964); SD = Standard deviation

4.3.6 Analysis of molecular variance

In order to partition the total variance into within and among regions, and within and among groups, the combined RAPD and ISSR profiles of *J. curcas* was subjected to AMOVA at two hierarchical levels (Table 4.5). AMOVA analysis showed that 86.9% of the total genetic variance resided between North-East and outside North-East among regions while the rest (13.1%) was attributed to the difference within-regions. Thus, AMOVA supported the results of Nei's gene diversity statistics and Shannon's information measure that there exists a high degree of genetic variance within regions. Among-group variability (between Arunachal Pradesh, Manipur, Mizoram, Meghalaya, Tripura, Assam and outside North-East) accounted for 72.09% of the molecular variation when carried out at the group level.

Table 4.5 - Analysis of molecular variance (AMOVA) of *J. curcas* using combined RAPD and ISSR data

Source of variation	d.f.	SSD	MSD	Variance component	Total Variance (%)
Between Outside N-E and N-E regions					
Within regions	1	55.5922	55.592	4.07	13.10
Among regions	31	836.8017	26.994	26.99	86.90
Between 7 groups					
Within groups	6	313.3457	52.224	8.62	27.91
Among groups	26	579.0482	22.271	22.27	72.09

d.f. = degree of freedom; SSD = Sum of Squared Deviations; MSD = Mean of Squared Deviations

4.3.7 Cluster and principal component analyses

The dendrogram based on RAPD profile using Jaccard's similarity matrix grouped the 33 *J. curcas* populations into four main clusters (Fig. 4.3). Cluster I and II were comprised of mixed populations while cluster III consisted of 4 populations from Assam. Cluster IV was specific to Meghalaya and Tripura. The dendrogram constructed from ISSR data comprised of four main groups (Fig. 4.4). Cluster I was the largest encompassing populations from mixed geographical origin. Clusters II, III and IV were specific to Assam, Arunachal Pradesh and Meghalaya respectively. The combined RAPD and ISSR data set based dendrogram dispersed the *J. curcas* populations into 4 distinct clades (Fig. 4.5). The populations from diverse geographical background (outside North-East, Manipur, Mizoram and all populations from Assam except IITJC15) formed cluster I. Clusters II and III were specific to their geographical origins (Arunachal

Pradesh and Assam respectively). The Meghalaya and Mizoram populations were grouped together in cluster IV.

The overall grouping patterns of the *J. curcas* populations delineated by RAPD and combined RAPD data based dendrograms were found be more or less similar while ISSR showed some variations when the major groups were considered. The populations from different eco-geographical regions outside North-East India grouped together in the sub-cluster IA of all the dendrograms. The formation of mixed sub-clusters in cluster I by populations from Manipur, Mizoram and Assam led to the inference that these populations shared similar genetic background and having common source of origin owing to the consequences of human interventions and translocations. Interestingly, IITJC15, the high seed yielding population from Assam, represented singly in all three clusters either maintaining distinct subgroup (IIIB in RAPD and IG in ISSR) or group (III in combined RAPD and ISSR). The strong association between Pasighat (IITJC5), Itanagar (IITJC6) and Naharlagoon (IITJC7) populations from Arunachal Pradesh (IIA in RAPD, III in ISSR and II in combined RAPD and ISSR) indicated that the genetic divergence is partially attributed to geographical origin. The formation of separate cluster (IV) by Mawhati (IITJC12) and Tura (IITJC13) populations and maintenance of maximum genetic distance with rest of the populations in all the dendrograms suggested that both genetic and geographical isolation as the reason behind high level of genetic heterogeneity within *J. curcas* populations of Meghalaya. The sole representative from Tripura, IITJC14, was found to have closest association with the genetically diverse populations of Meghalaya in RAPD and combined RAPD and ISSR dendrograms.

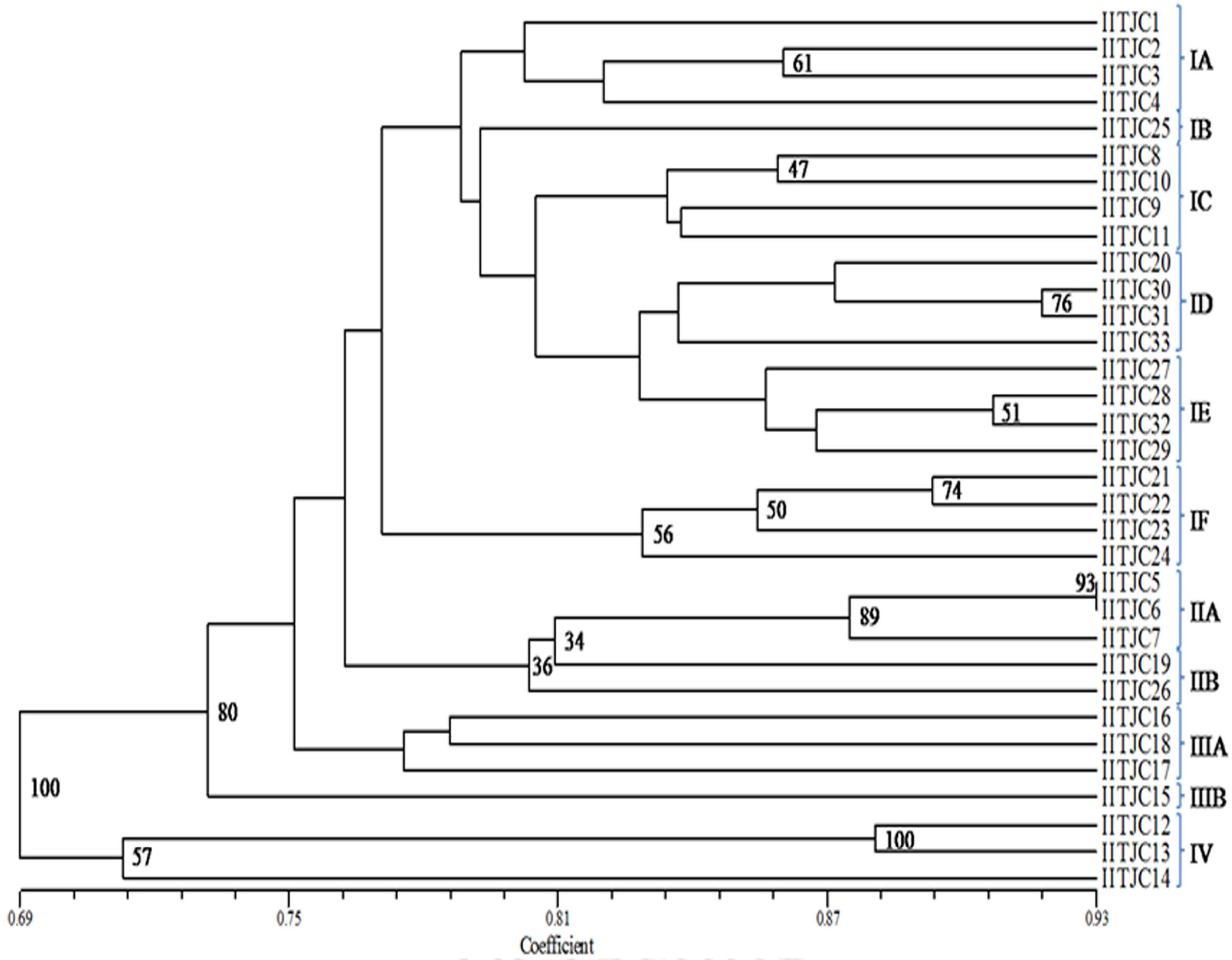


Fig. 4.3 Genetic relationship among 33 *J. curcas* populations obtained by RAPD analysis based on UPGMA based cluster analysis

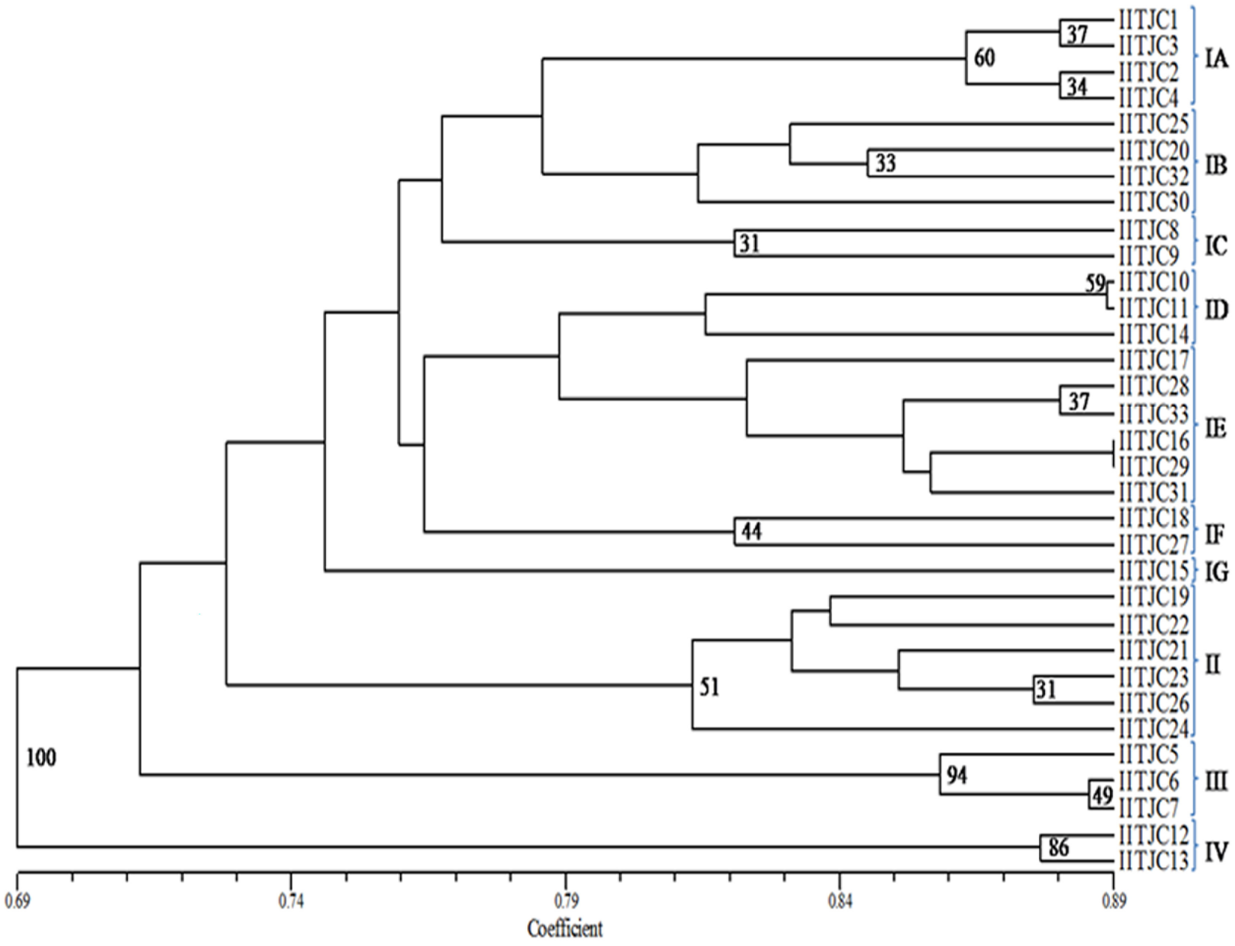


Fig. 4.4 Genetic relationship among 33 *J. curcas* populations obtained by ISSR analysis based on UPGMA based cluster analysis

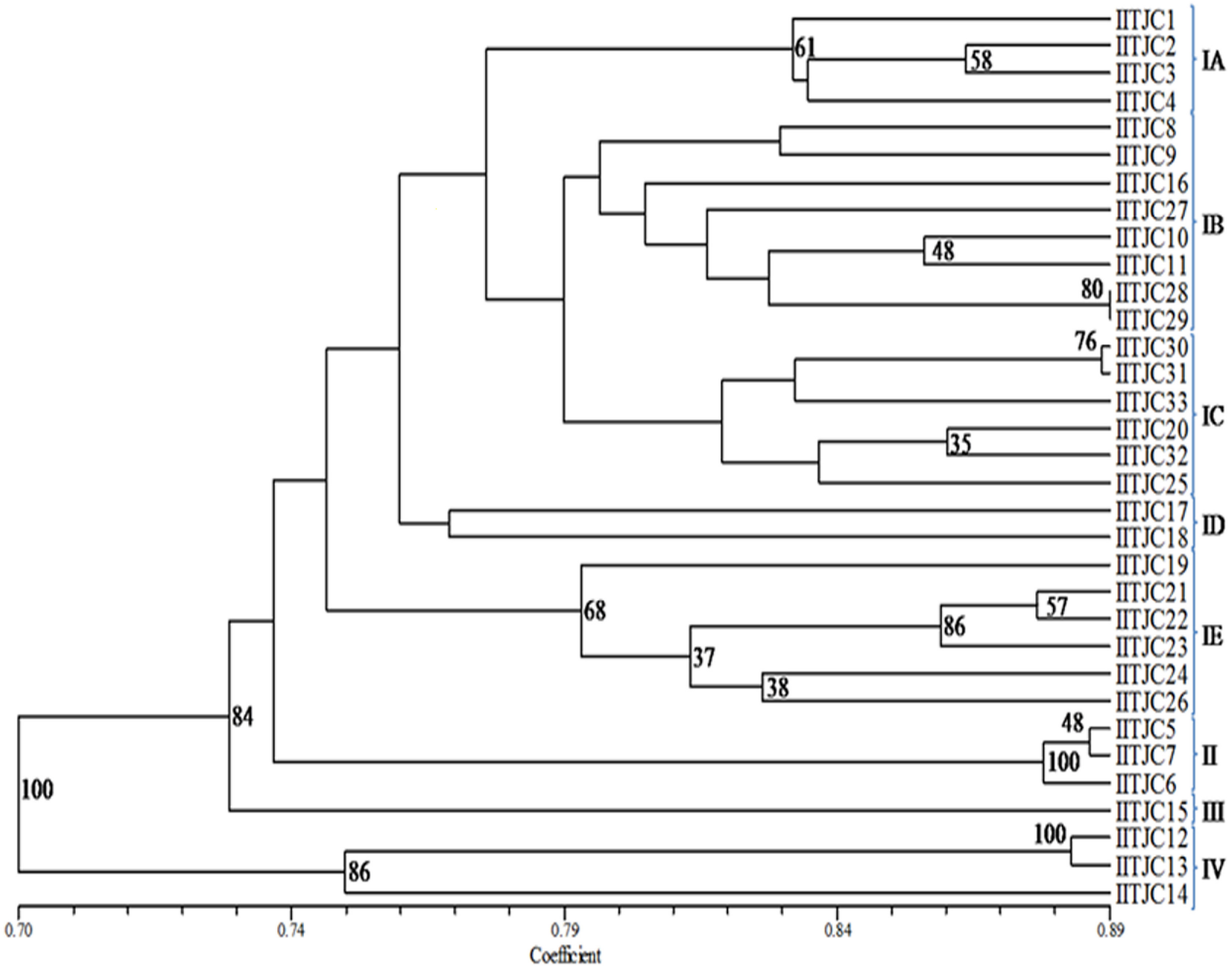


Fig. 4.5 Genetic relationship among 33 *J. curcas* populations obtained by combined RAPD and ISSR analysis based on UPGMA based cluster analysis

The structurization of major groups of RAPD (Fig. 4.6), ISSR (Fig. 4.7) and combined RAPD and ISSR (Fig. 4.8) based PCA plots was comparable with the cluster analysis result. It was observed that some populations from Assam having high genetic similarity index with populations from outside North-East merged together in sub-group IA instead of forming separate sub-groups as in cluster analysis. On contrary, the grouping patterns of populations from Arunachal Pradesh (IITJC5, IITJC6 and IITJC7), Meghalaya (IITJC12 and IITJ 13) and Tripura (IITJC14) in PCA plots were in perfect corroboration with cluster analysis results.

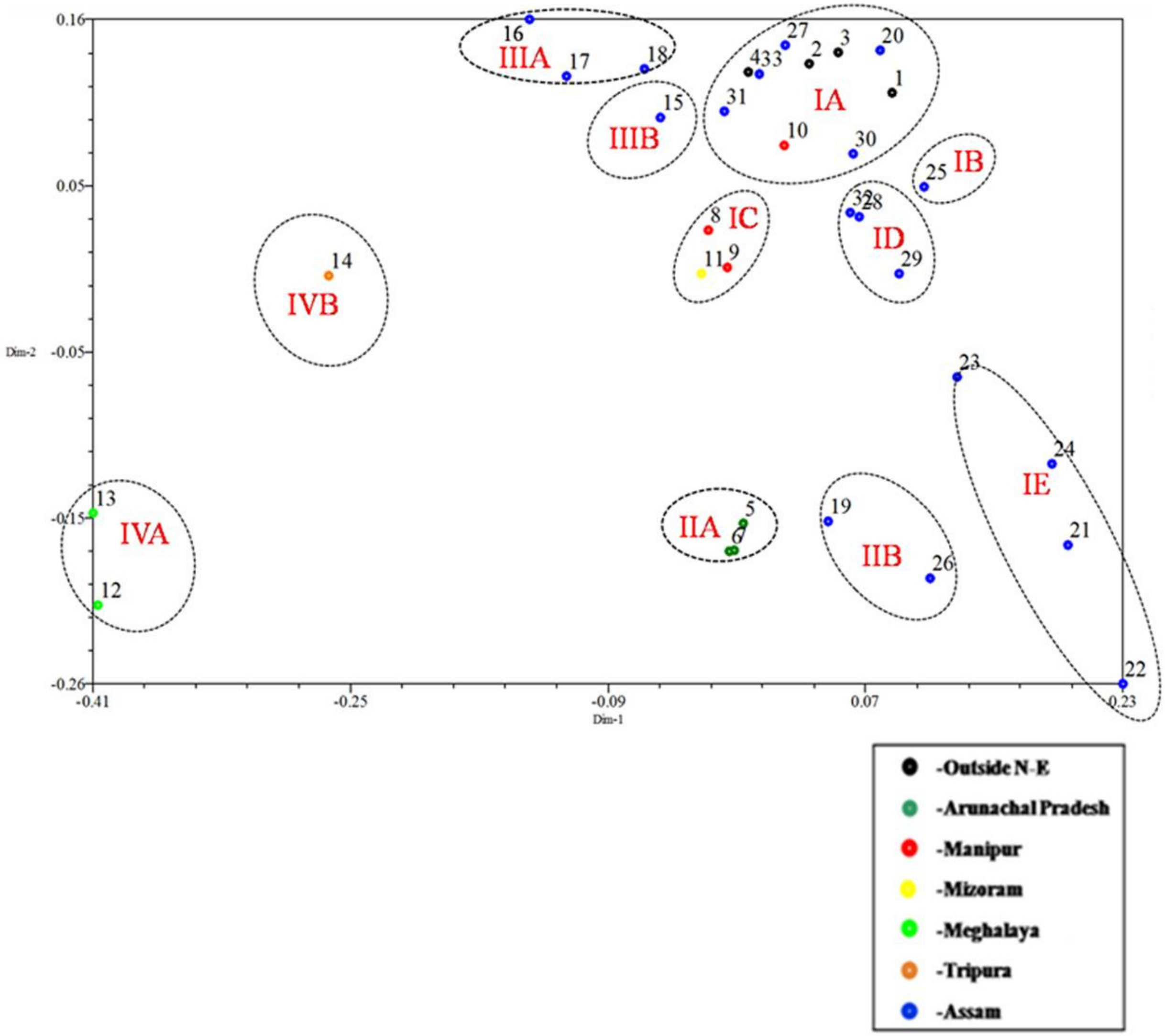


Fig. 4.6 Genetic relationship among 33 *J. curcas* populations obtained by RAPD analysis based on Principal Component Analysis

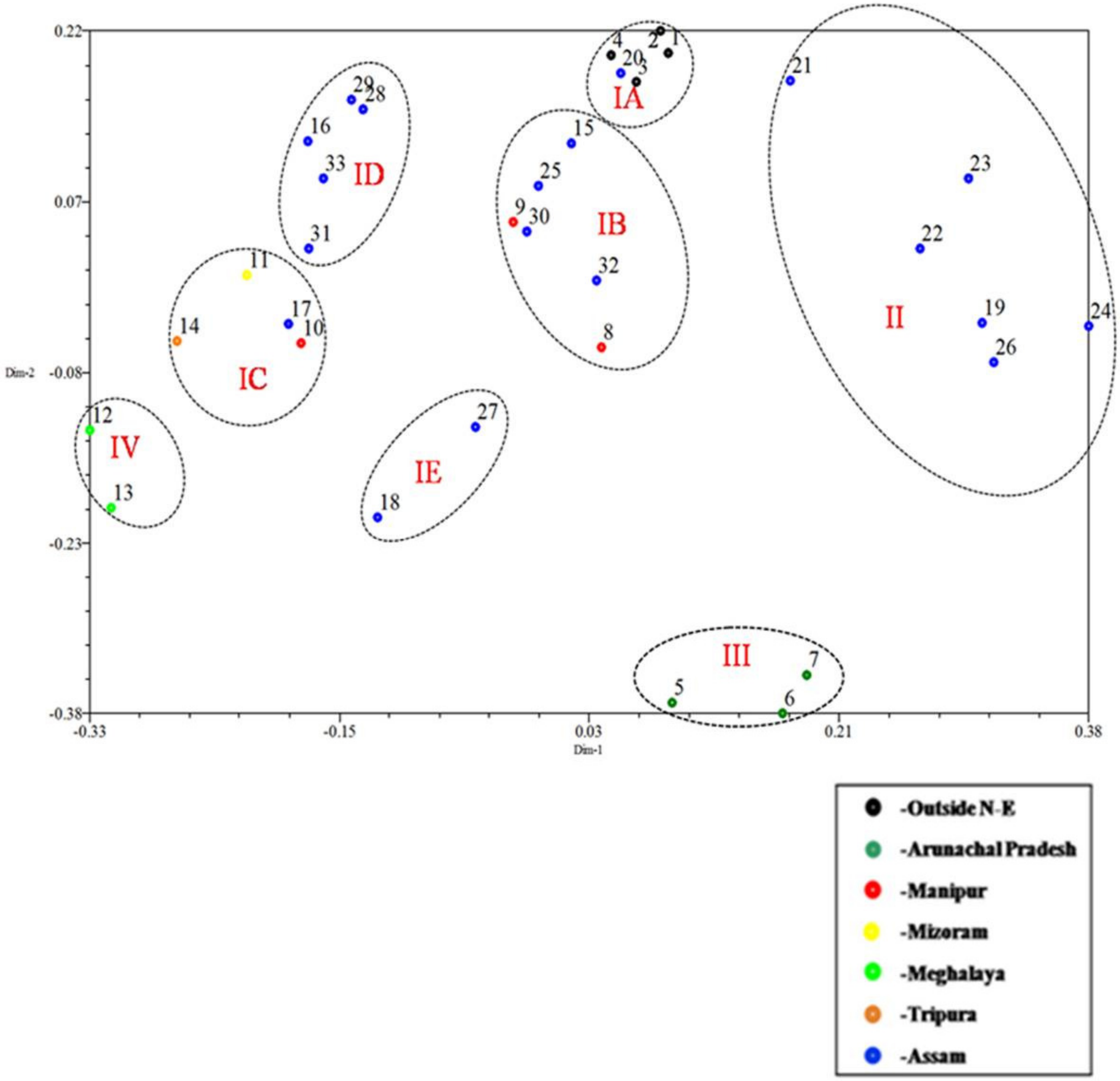


Fig. 4.7 Genetic relationship among 33 *J. curcas* populations obtained by ISSR analysis based on Principal Component Analysis

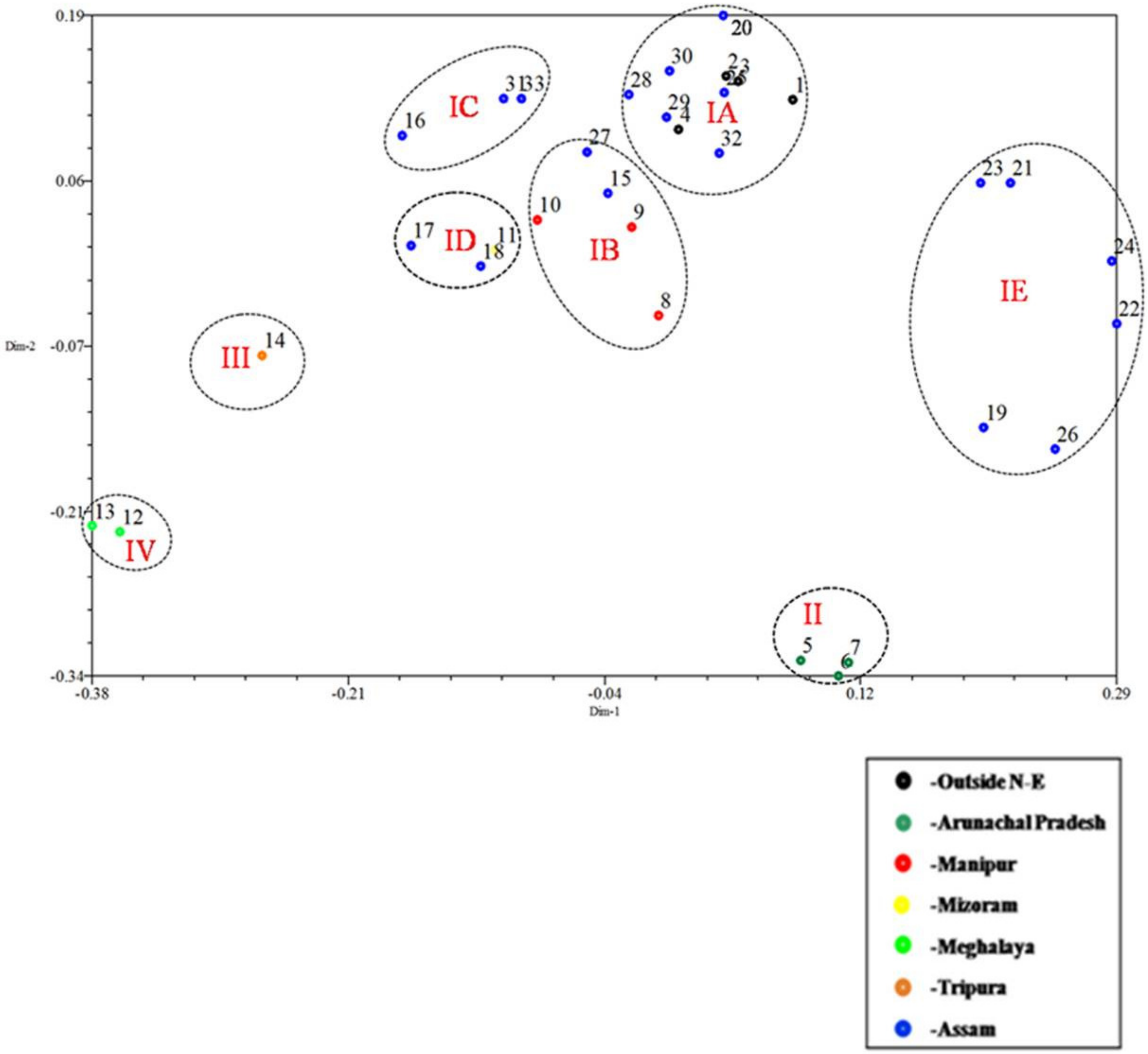


Fig. 4.8 Genetic relationship among 33 *J. curcas* populations obtained by combined RAPD and ISSR analysis based on Principal Component Analysis

The molecular data based cluster and principal component analysis indicated presence of significant genetic divergence amongst *J. curcas* populations of North-East India. The high level of geographical isolation observed in case of Arunachal Pradesh, Meghalaya and Tripura populations was consistent with the findings of Ranade et al. (2008), which inferred that longer history of geographical isolation of North-East populations to be the main reason behind the preservation of greater genetic heterogeneity. However, the previous findings (Kumar et al. 2011; 2013) failed to depict any geographical isolation or ecological differentiations among North-East populations. This contradiction is likely due to the limited number of populations (only Assam and Meghalaya) and less number of primers used in previous studies was insufficient for finding the true scenario of genetic relationships among *J. curcas* populations of North-East India. The single population from Assam, IITJC15, which emerged as genetically diverse from the other 18 populations from Assam, was considered as discernible. The observation that Arunachal Pradesh (IITJC5, IITJC6 and IITJC7), Meghalaya (IITJC12 and IITJC13) and Tripura (IITJC14) populations have maintained separate groups at significant genetic distance from the remaining populations suggested that these populations can play substantial role in genetic enhancement and broadening of the *J. curcas* genetic resources.

The monomorphic data generated by curcin-P2 primer pairs for all 33 *J. curcas* populations were not considered for cluster analysis and principal component analysis.

4.3.8 Correlation of genetic diversity matrices

Mantel matrix correspondence test revealed a moderate level of positive correlation (r = 0.58) between RAPD and ISSR based similarity matrices (Fig. 4.9A). The variations in clade structure of RAPD and ISSR based cluster analysis was a reflection of the moderate correspondence observed in Mantel's correlation test. The observed disparity was probably due to the different regions of genomic DNA targeted by RAPD and ISSR markers. A highly positive correspondence (r = 0.92) between RAPD and combined RAPD + ISSR datasets was observed (Fig. 4.9B). High congruency (r = 0.84) was also observed between ISSR and combined RAPD+ISSR matrices (Fig. 4.9C).

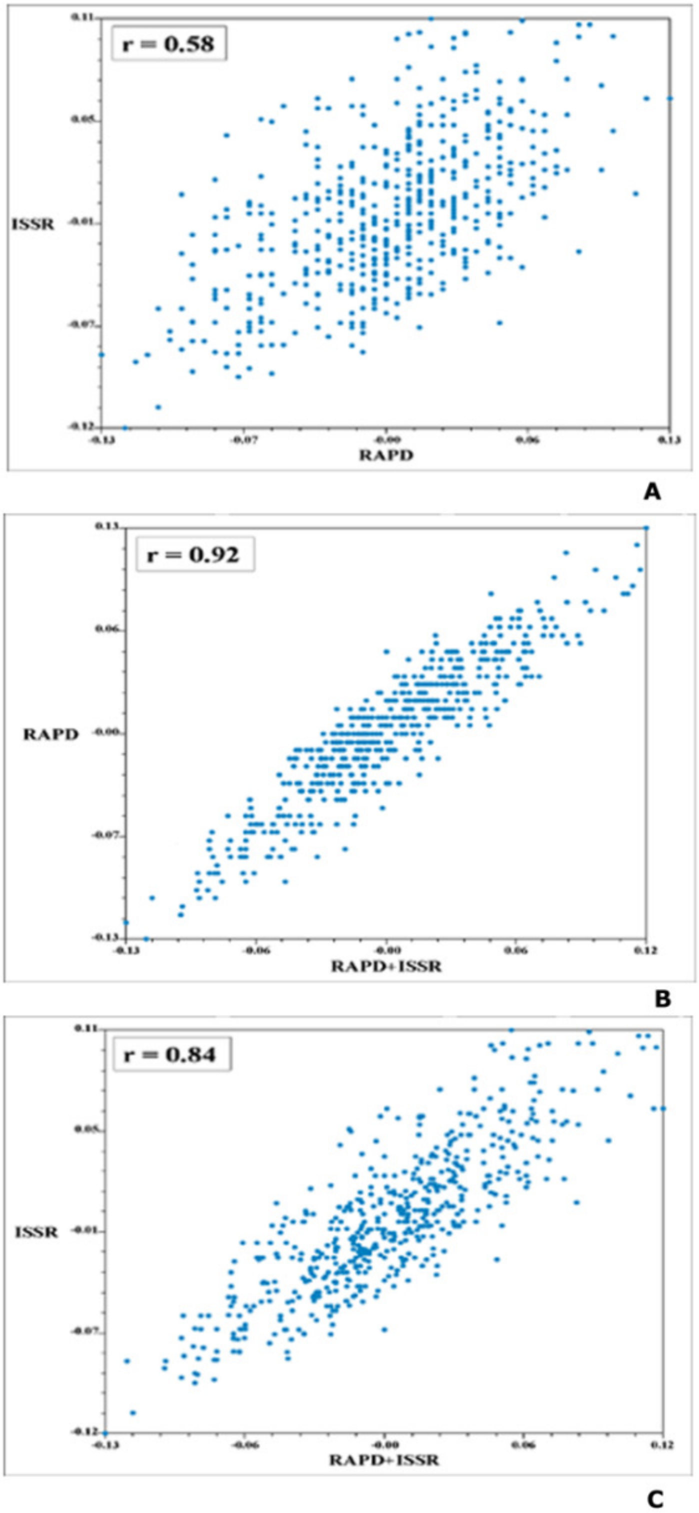


Fig. 4.9 Mantel matrix correlation set for three random marker sets

4.4 Conclusion

The present investigation represents the first successful attempt of characterization of genetic diversity in *J. curcas* populations of North-East India using random and gene-specific molecular markers. This study suggested that (1) North-East Indian populations of *J. curcas* are more diverse than populations from other parts and therefore particularly valuable from the perspective of broadening the genetic diversity in *J. curcas* germplasm. However, the number of outside North-East populations considered was less. In future, studies will be conducted by including more *J. curcas* populations from outside North-East. This will help in further assessment of the extent of *J. curcas* genetic diversity between North-East and outside North-East populations. (2) There is a moderate association between geographic isolation and genetic heterogeneity among *J. curcas* populations of North-East India. IITJC5, IITJC6 and IITJC7 from Arunachal Pradesh, IITJC12 and IITJC13 from Meghalaya, IITJC14 from Tripura and IITJC15 from Assam were identified as genetically diverse. All seven genetically diverse populations were advanced for further investigation related to estimation of *J. curcas* seed phorbol ester content.

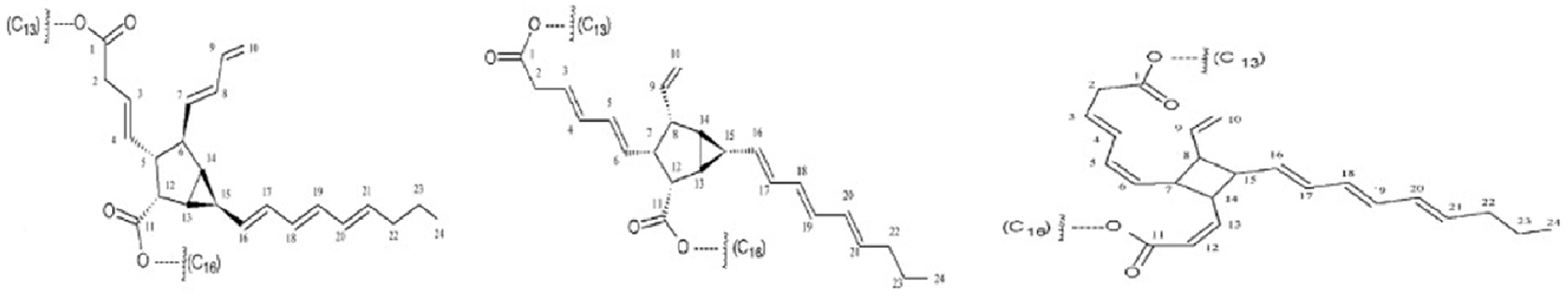
Chapter 5

Biochemical characterization of *J. curcas* for identification of elite populations having low phorbol ester content

5.1 Introduction

J. curcas, a member of the Euphorbiaceae (spurge family) is a perennial oil seed plant. *J. curcas* seeds contain high amounts of oil (30-50% by seed weight) and have been considered as a sustainable fuel alternative in tropical and sub-tropical countries (Pramanik 2003; Becker and Makkar 2008). However hitherto, the net profit earned from *J. curcas* derived biofuel is insufficient. Exploitation of by-products obtained during *J. curcas* biodiesel production is an effective way to improve the overall economic return (Prasad et al. 2012). Extraction of oil from *J. curcas* seeds generates large quantity of seed cake as by-product with an average rate of 500 g cake per kg of seed used (Zanzi et al. 2008). The protein-rich (~65% on a dry matter basis) seed cake comprises of high concentrations of essential amino acids (except lysine) and has a nutritional value comparable to soybean meal (Francis et al. 2013). Thus the efficacious utilization of the left-over seed cake as an animal feed ingredient will substantially enhance the economic return from *J. curcas* derived biodiesel (Makkar and Becker, 1997). However, presence of high concentrations of toxic constituents and anti-nutritional agents like curcins, saponins, lectins, phytate, protease inhibitors and phorbol esters are the impediments behind successful exploitation of the seed cakes as fodder. Furthermore, toxic *J. curcas* seeds, seed cakes and seed oil can cause health risk for farmers and bioprocess workers (Achten et al. 2007). Metabolomic profiles of toxic and non-toxic *J. curcas* seeds have revealed the fact that presence of co-carcinogenic phorbol esters, a group of tetracyclic diterpenoids, is the main reason behind *J. curcas* seed-toxicity (Makkar et al. 1998).

Phorbol esters are a group of naturally occurring compounds which are widely distributed in plants belonging to the families Euphorbiaceae and Thymelaeceae (Evans and Taylor 1983). Phorbol esters are described as “polycyclic compounds in which two hydroxyl groups on neighboring carbon atoms are esterified to fatty acids” (Goel et al. 2007). The phorbol ester derivatives are potent tumor promoters and often cause biological effects like inflammations and skin irritation (Haas and Mittelbach 2000). Presence of at least six diester derivatives of 12-deoxy-16-hydroxyphorbol, *Jatropha* factors C1-C6 (Fig. 5.1), has been reported in *J. curcas* seeds (Haas et al. 2002).



Jatropha factor C1

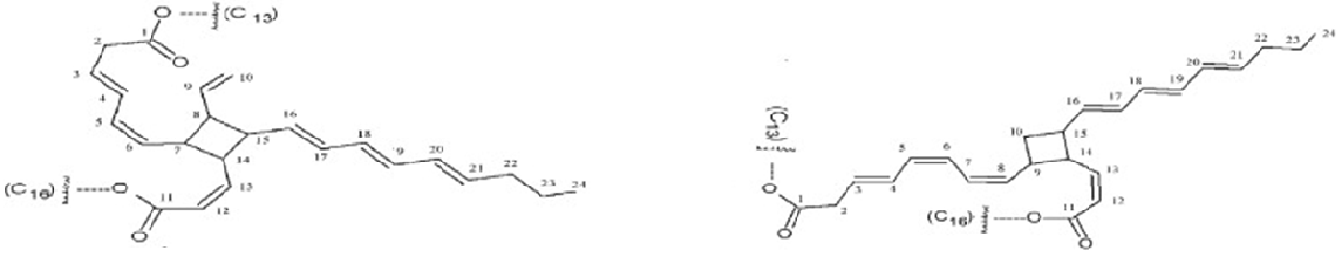
(A)

Jatropha factor C2

(B)

Jatropha factor C3/C4

(C)



Jatropha factor C5

(D)

Jatropha factor C6

(E)

Fig. 5.1 Phorbol ester derivatives present in *J. curcas* seeds. *Jatropha* factors: C1–C6 (A–E). C3 and C4 are epimers (Source: Adapted with permission from Haas et al. 2002, copyright 2002, American Chemical Society)

Several studies have affirmed the fact that with the exception of the ‘nontoxic’ Mexican provenances; all other *J. curcas* seeds contain high quantity of phorbol esters (Makkar et al. 1997; Gubitz et al. 1999). However, the concentration of phorbol esters in *J. curcas* seeds varies greatly between toxic populations collected from different ecogeographical regions (Liu et al. 2013). It was found that phorbol ester concentration in toxic *J. curcas* populations generally ranges from 0.87 to 3.32 mg/g of the kernels (Makkar et al. 1997).

Phytochemical characterization of *J. curcas* based on phorbol ester concentration may lead to the identification of elite and diverse populations from North-East India having comparatively low concentrations of phorbol esters in seed. Morphometric and molecular characterizations (Chapter 3 and Chapter 4) have already revealed presence of diversity among North-East Indian populations. In this chapter, biochemical characterization of *J. curcas* populations was conducted with an objective to estimate phorbol ester concentration of twelve *J. curcas* populations from North-East India, which were selected as elite and diverse on the basis of morphometric and molecular characterization (Chapter 3 and Chapter 4).

5.2 Materials and methods

5.2.1 Materials

One population from Tripura, two populations from Meghalaya, three populations from Arunachal Pradesh and six populations from Assam were included in the study (Table 5.1). Mature and sun-dried *J. curcas* seeds collected from twelve North-East Indian populations were used for phorbol ester estimation. The external standard, Phorbol 12-myristate-13-acetate (PMA, purity ≥ 99%), was procured from Sigma–Aldrich (USA) and Formic acid from Fluka (Switzerland). All other chemicals (Methanol, Acetonitrile and Water) were bought from Merck Pvt. Ltd., (India). All chemicals used for phorbol ester extraction and HPLC analysis were of HPLC and analytical grade.

Table 5.1 - ***J. curcas*** **populations for phorbol ester analysis**

Sl. No.	Population No.	Location of collection	Morphometric trait	Molecular Trait
1	IITJC5	Pasighat (East Siang, Arunachal Pradesh)	-	Genetically diverse
2	IITJC6	Itanagar (Papum Pare, Arunachal Pradesh)	-	Genetically diverse
3	IITJC7	Naharlagoon (Papum Pare, Arunachal Pradesh)	High 100 seed weight	Genetically diverse
4	IITJC12	Mawhati (Ri Bhoi, Meghayalaya)	-	Genetically diverse
5	IITJC13	Tura (West Garo Hills, Meghalaya)	-	Genetically diverse
6	IITJC14	Agartala (West Tripura, Tripura)	-	Genetically diverse
7	IITJC15	Amingaon (Kamrup, Assam)	High total seed yield, low male: female flower ratio, Morphologically diverse	Genetically diverse
8	IITJC19	Tezpur (Sonitpur, Assam)	Morphologically diverse	-
9	IITJC21	Teok (Jorhat, Assam)	Morphologically diverse	-
10	IITJC22	Makum (Tinsukia, Assam)	Morphologically diverse	-
11	IITJC24	Numaligarh (Golaghat, Assam)	High plant height and canopy spread, Morphologically diverse	-
12	IITJC28	Bokajan (Karbi Anglong, Assam)	Maximum collar diameter	

5.2.2 Extraction of phorbol esters from *J. curcas* seeds

The testa was removed from *J. curcas* seeds. 1 g of *J. curcas* seed kernel from each population was ground to a fine powder using morter and pestle. The ground kernel was transferred to a 30 ml polypropelene Oak Ridge centrifuge tube. Phorbol ester was extracted from the kernel powder with 10 ml of HPLC grade methanol for 30 mins at 200 rpm in orbital shaker. The mixture was centrifuged at 5000 rpm for 10 mins at 4 °C. The supernatant was stored. The kernel residue was re-extracted with 10 ml of methanol for two times. The supernatants from all three extractions were combined. The 30 ml of combined supernatant was concentrated at 40 °C under reduced pressure in a rotary evaporator (Eyela, Japan) until the final volume reached to 2 ml.

5.2.3 Preparation of standard phorbol ester

The external standard, Phorbol-12-myristate-13-acetate (PMA), was dissolved in methanol and diluted to working concentrations of 5 µg, 10 µg, 15 µg and 20 µg respectively in 20 µL of injection volumes. The standard solutions were filtered through 0.45 µM SPARTAN (Whatman, Germany) syringe filters prior to injection. The standard solutions were analyzed by Perkin Elmer PE 200 series High Pressure Liquid Chromatography (HPLC) system using a Shodex (Germany) Analytical C18 reverse phase column. The peak areas were quantified using Total Chrom Workstation Ver. 6.2.0 (Perkin Elmer Inc., USA). A calibration curve of good linearity (R^2>99%) was prepared by plotting the peak areas of PMA obtained from HPLC analysis vs. known concentrations (Fig. 5.4). The calibration curve was used to quantify total phorbol ester content in *J. curcas* seed samples.

5.2.4 Analysis of phorbol esters

5.2.4.1 HPLC analysis

The concentrated phorbol ester sample solution was filtered through 0.45 µM SPARTAN syringe filter. A sample volume of 20 µl was analyzed by Perkin Elmer PE 200 series HPLC system using a Shodex Analytical C18 reverse phase column (250 x 4.6 mm I.D., particle size 5.6 µm, pore size

110 Å, carbon load 17%). Elution solvents were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. All solvents were filtered and degassed before HPLC analysis. The flow condition used was as follows: 40% of solvent A and 60% of solvent B for 5 min, linear gradient to 75% B in next 20 min; increase B to 100% in next 30min. The total run time required for analyzing one sample was 55 min. After completion of each run, the column was adjusted to the starting condition, i.e. 40% of solvent A and 60% of solvent B. The UV detector wavelength was set at 280 nm according to the λ_{max} of UV absorbance of phorbol esters (ref). The separation was performed with a flow rate of 1 ml/min at room temperature (23 °C). Same experimental conditions were maintained for twelve *J. curcas* samples and PMA standard. Five major peaks corresponding to phorbol ester fractions were obtained for each *J. curcas* sample. The chromatography package Total Chrom Workstation Ver. 6.2.0 was used to quantify peak areas of *J. curcas* phorbol esters. The areas of the peaks obtained at 280 nm were integrated. Phorbol ester concentrations of the 12 samples were calculated from integrated peak areas with reference to the peak area and concentration of the standard (PMA). HPLC analysis for each *J. curcas* population was conducted in triplicate. The phorbol ester concentrations are presented as mean ± standard error. The mean phorbol ester content of the North-East states (Arunachal Pradesh, Assam and Meghalaya) were also calculated. However, the mean phorbol ester content was not estimated for Tripura which was having only one representative.

5.2.4.2 MS analysis

In order to confirm the *Jatropha* factors, mass spectrometric analysis was conducted in negative ion mode ($[M\text{-}H]^-$) using an Agilent-Q-TOF 6500 mass spectrometer. All datas were acquired and analyzed by Mass Hunter software (Agilent Technologies, USA).

5.3 Results and discussion

5.3.1 Detection of *J. curcas* phorbol ester factors by HPLC analysis

During HPLC analysis, a single peak corresponding to PMA, the external standard, was obtained at 6.35 min. For *J. curcas* phorbol ester samples, five well separated peaks corresponding to six *J. curcas* phorbol ester factors C1, C2, C5, C3-C4 and C6 respectively were obtained at retention times ranging from 2.16 to 7.7 min for each population. The phorbol ester factors C3 and C4 are epimers and were not separated by HPLC. The *Jatropha* factors C1-C6 have the basic structure of 12-deoxy-16-hydroxyphobol. They differ from each other in side chains (Fig. 5.1) (Haas et al. 2002). The HPLC chromatograms of phorbol esters extracted from *J. curcas* populations IITJC12, IITJC7 and IITJC15 has been delineated in Fig. 5.2A, 5.2B and 5.2C respectively. Fig. 5.2D shows the HPLC chromatogram of the standard (PMA).The HPLC chromatograms obtained for *J. curcas* phorbol esters were consistent with previous report (Liu et al. 2013).

5.3.2 Confirmation of *J. curcas* phorbol ester factors by MS analysis

The molecular weight of *J. curcas* phorbol esters were confirmed by subsequent mass spectrometry. Mass spectrometric analysis of phorbol esters were performed in both negative and positive ion modes. However, it was observed that peak intensities of the standard (PMA) and *J. curcas* phorbol esters are stronger in negative ion mode. As depicted in Fig. 5.3A to Fig. 5.3E, protonated molecular ions of m/z = 710.38 was obtained for all five peaks at negative ion mode ($[M-H]^-$) in all twelve North-East populations of *J. curcas*. Thus, the accurate mass of 711.38 corresponding to phorbol esters was obtained for the five phorbol ester peaks. Fig. 5.3F represents the mass spectrum of the external standard, PMA. The parent fragment of m/z=615.8 corresponding to PMA was detected under negative ion mode. The results obtained in mass spectrometric analysis were in concordance with previous report (Ohtani et al. 2012). In addition to the parent ions, other fragmentations corresponding to the quasimolecular ions of phorbol esters, such as, m/z 691.3, 693.3, 695.1 and 709.2, were also detected in our mass spectrum dataset. Similar fragmentation of phorbol esters derivatives in mass spectra were also reported in previous reports (Ohtani et al. 2012; Goel et al. 2007; Vogg et al. 1999).

Thus from the HPLC chromatograms and mass spectra it was inferred that six derivatives of phorbol esters are present in seed samples of all twelve *J. curcas* populations from North-East India.

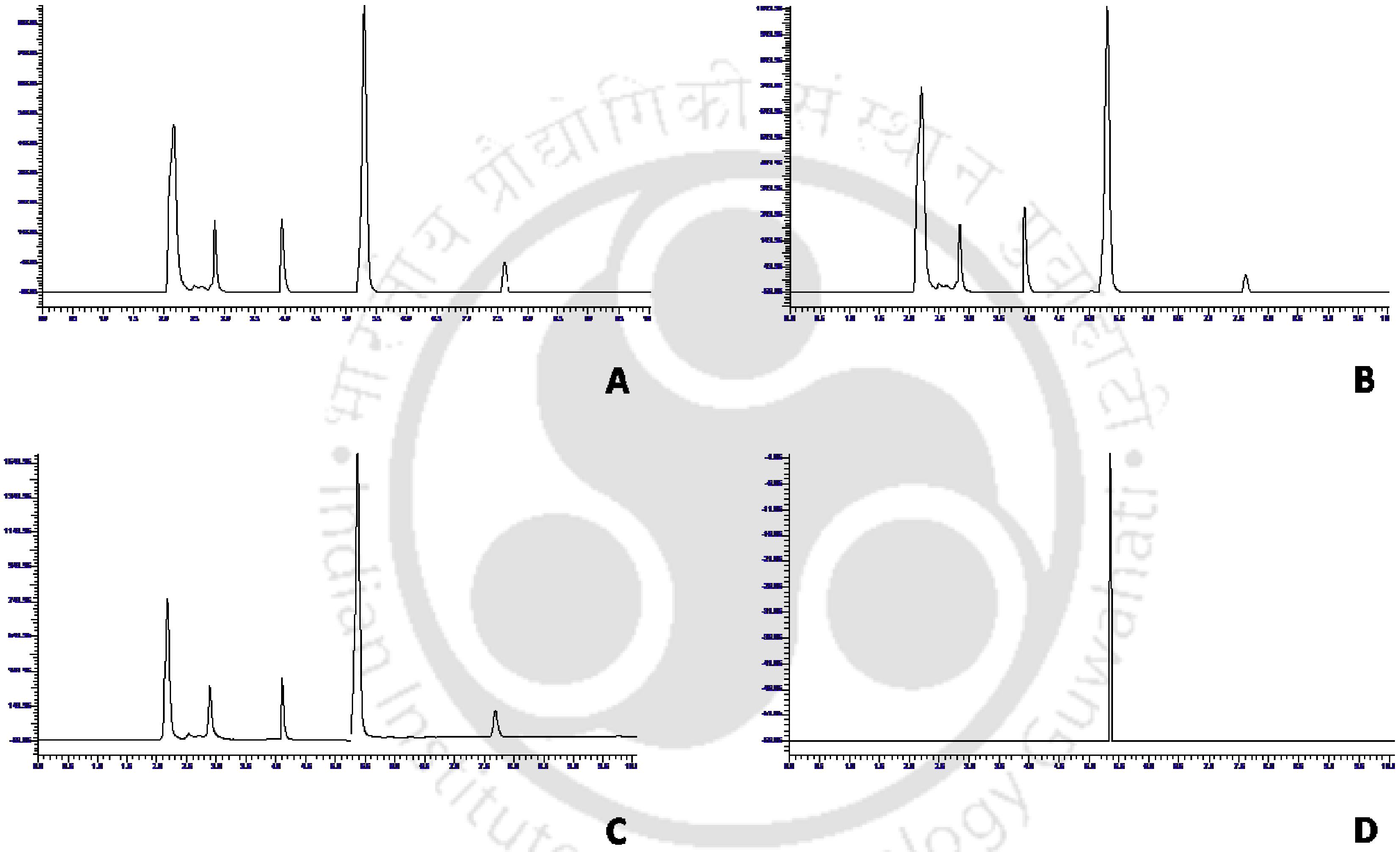


Fig. 5.2 HPLC chromatogram of (A-C) *J. curcas* seed phorbol esters (A) IITJC12, (B) IITJC7, (C) IITJC15 and (D) standard (phorbol-12-myristate-13-acetate)

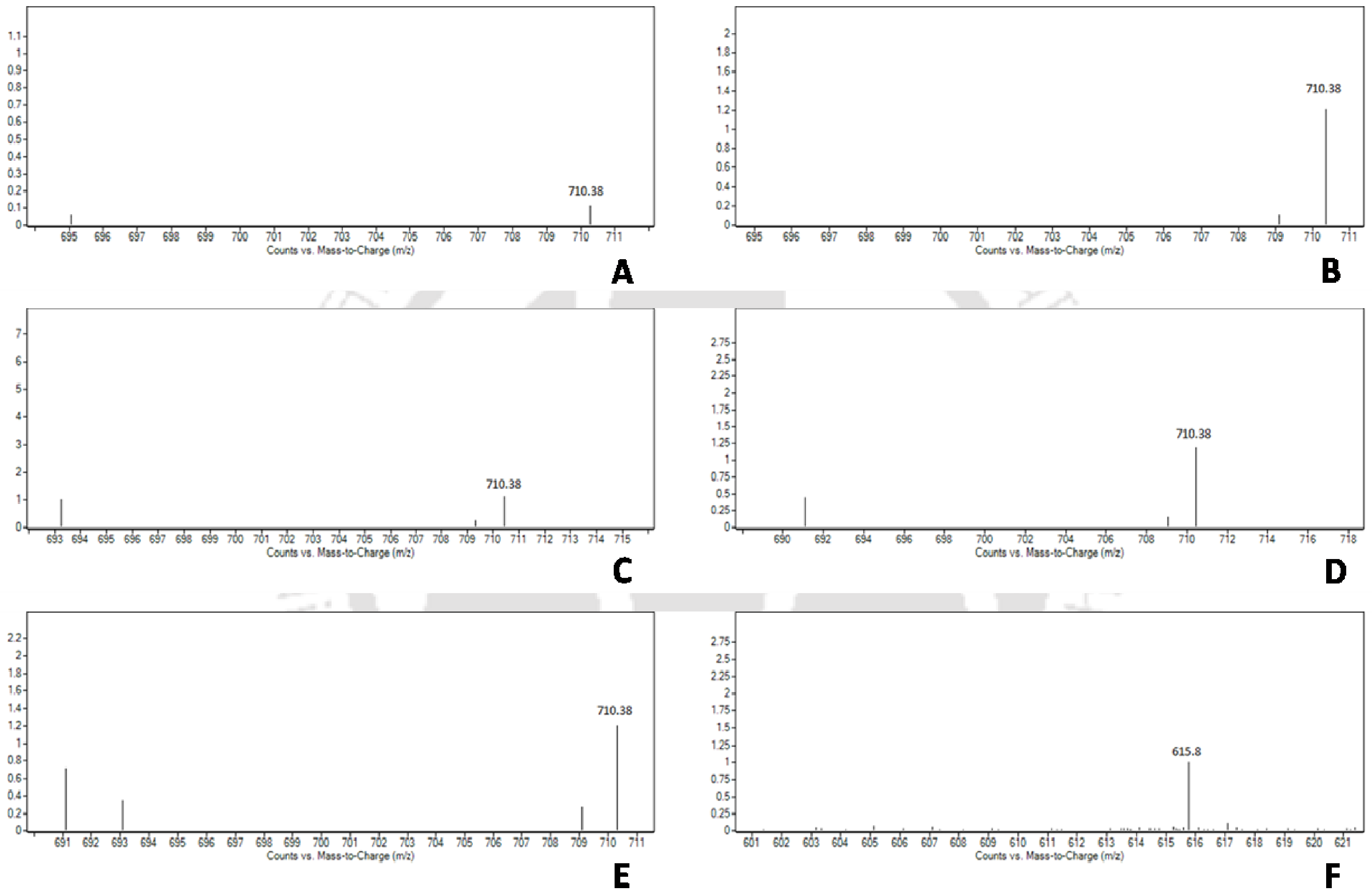


Fig.5.3 Mass spectrum of (A-E) *J. curcas* phorbol ester derivatives and (F) standard (phorbol-12-myristate-13-acetate) under negative ion mode

5.3.3 Quantification of phorbol esters in *J. curcas* populations from North-East India

The phorbol ester concentration in twelve *J. curcas* populations were estimated from the HPLC calibration curve of PMA (Fig. 5.4).The results indicated that total phorbol ester content varied significantly (1.02 mg/g to 2.75 mg/g) among North-East populations of *J. curcas* (Table 5.2, Fig. 5.5). It has been previously reported that total phorbol ester content in toxic *J. curcas* seeds varies within the range of 0.87 mg/g to 3.32 mg/g (Makkar et al. 1997). When the results obtained in this study were compared with previous report, it was revealed that *J. curcas* seeds from Mawhati (IITJC12) and Naharlagoon (IITJC7) contain comparatively low concentrations of total phorbol ester (1.02 mg/g and 1.15 mg/g respectively). However, two populations from Assam, Teok (IITJC21) and Makum (IITJC22), were found to contain high concentrations of phorbol ester (2.54 mg/g and 2.75 mg/g respectively). It was observed that except IITJC15, all populations from Assam are having high phorbol ester content (2.05 mg/g to 2.75 mg/g).

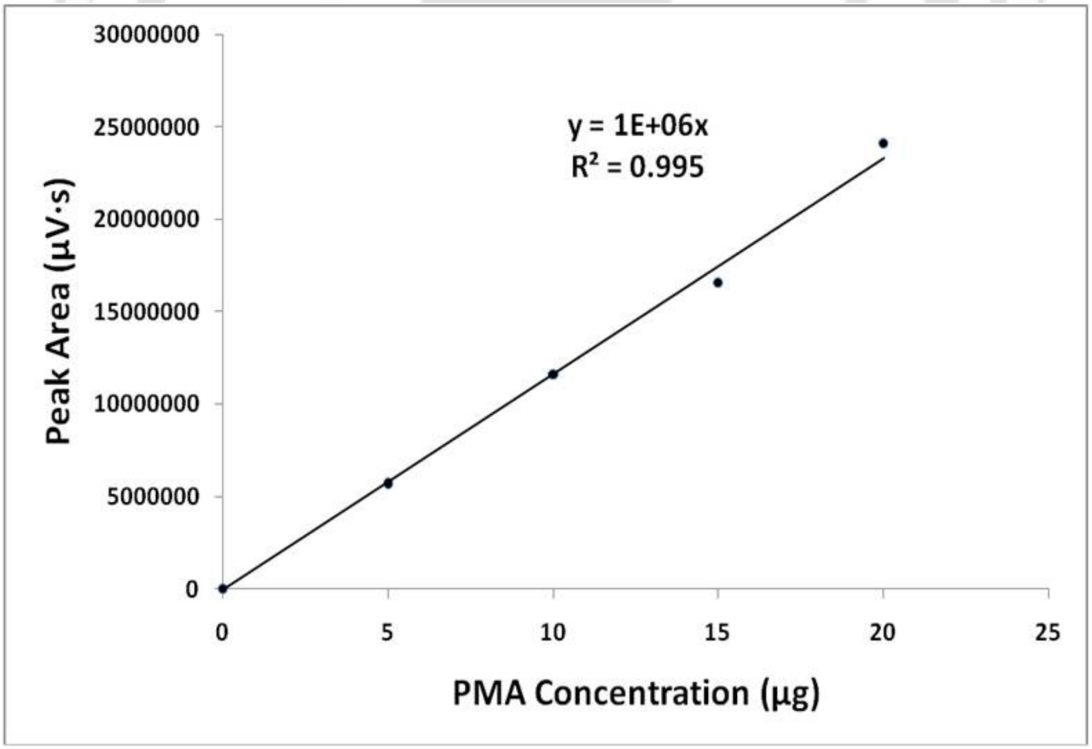


Fig.5.4 HPLC calibration curve of the standard (Phorbol 12-myristate 13-acetate)

For understanding that whether the variation in total phorbol ester content was correlated with geographic locations of the seeds, the ecogeographical location wise mean phorbol ester content in *J. curcas* seeds was calculated (Table 5.2). It was elucidated that *J. curcas* populations from geographically isolated hilly states of Meghalaya and Arunachal Pradesh are having comparatively low concentrations of seed phorbol ester (1.36 mg/g and 1.62 mg/g respectively). On the other hand, the populations from Assam showed high concentrations of phorbol ester (2.2 mg/g). Thus, from the results it was inferred that the variation in *J. curcas* phorbol ester concentration is related to the ecogeographical location of the populations. The results obtained were in concordance with previous study, where, region specific variation in *J. curcas* phorbol ester content was observed (Liu et al. 2013).

Table 5.2 – Total phorbol ester content in *J. curcas* populations

Sl. No.	***J. curcas* populations**	**Total phorbol ester content (mg/g of seed kernel)**	**Location**	**Mean phorbol ester content (mg/g of seed kernel)**
1	IITJC5	1.91±0.03	Arunachal Pradesh	1.62
2	IITJC6	1.81±0.02		
3	IITJC7	1.15±0.01		
4	IITJC12	1.02±0.01	Meghalaya	1.36
5	IITJC13	1.69±0.02		
6	IITJC14	2.26±0.04	Tripura	-
7	IITJC15	1.38±0.02	Assam	2.2
8	IITJC19	2.36±0.05		
9	IITJC21	2.54±0.05		
10	IITJC22	2.75±0.04		
11	IITJC24	2.05±0.04		
12	IITJC28	2.10±0.02		
Coefficient of Variation (%)		0.27		

± represents standard error of means

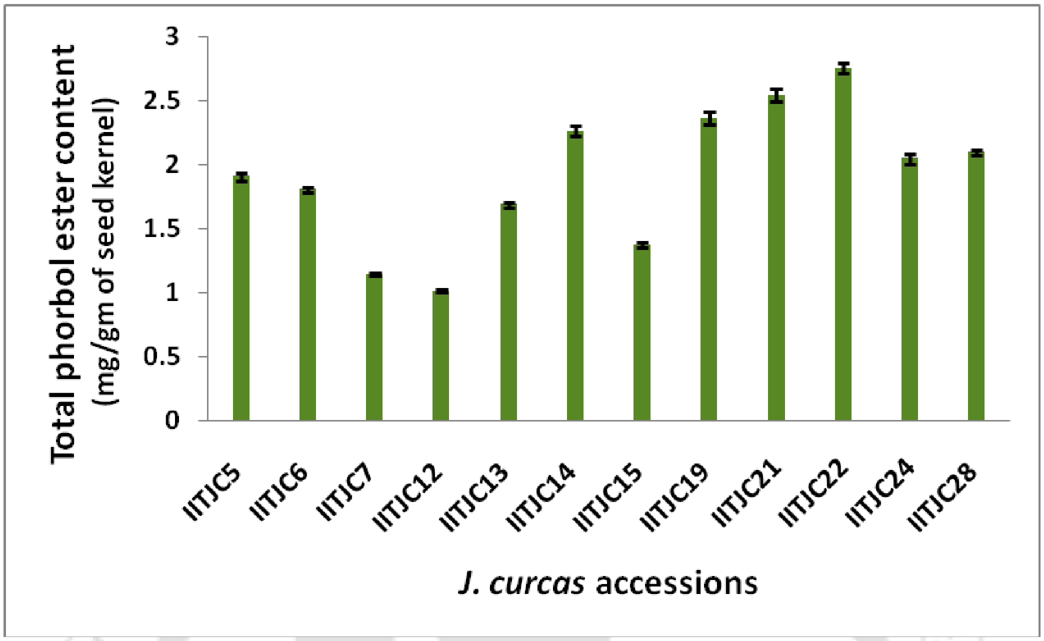


Fig.5.5 Phorbol ester content of *J. curcas* seeds collected from North-East India

5.4 Conclusion

The present study clearly depicted existence of considerable variation in phorbol ester content among *J. curcas* populations from North-East India. Furthermore, region specific variation in phorbol ester was observed among North-East populations. The presence of at least six *Jatropha* factors was affirmed in all populations from North-East India by high pressure liquid chromatography and mass spectrometry. Three *J. curcas* populations, IITJC12 (1.02 mg/g of seed kernel), IITJC7 (1.15 mg/g of seed kernel) and IITJC15 (1.38 mg/g of seed kernel), from Meghalaya, Arunachal Pradesh and Assam respectively were identified as low phorbol ester containing populations. Previous studies have already confirmed IITJC7 and IITJC15 as populations having high seed yield traits (Chapter 3) and all three populations as genetically diverse (Chapter 4). Thus it can be concluded that the three North-East Indian populations identified as elite, diverse and less toxic by comprehending morphometric, molecular and biochemical traits can be efficaciously exploited in future *J. curcas* breeding programs.

Chapter 6

Studies on genetic distance of *J. curcas* with *J. podagrica* and *J.* gosssypifolia

6.1 Introduction

The genus *Jatropha* comprised of about 175 old and new world woody species, most of which are having medicinal and economical significance. It is native to Central America and has been later dispersed to Africa and Asia and naturalized therein (Dehgan 1984). Of the 175 known species, only few species have been reported in India of which *J. curcas*, *J. podagrica* and *J. gossypifolia* are of immense importance.

J. curcas has received considerable global attention as a potential biodiesel plant. The gregarious shrub *J. gossypifolia* (bellyache bush), a relatively less known member of *Jatropha* family, is known to bear more number of fruits than *J. curcas* and is also highly tolerant to soil salinity (Dagar et al. 2006). Furthermore, the seed oils of *J. gossypifolia* (42.2 MJ/Kg) have relatively higher energy content than *J. curcas* (39.8–41.8 MJ/Kg). The caudiciform plant *J. podagrica* (bottleplant shrub) often promoted as ornamental plant, bears seeds perennially (Basha and Sujatha 2009). The fact that *Jatropha* species are highly xenogamous, has opened ample opportunities to plant breeders for improvement of *J. curcas* for continuous fruit-bearing, improved fuel traits and wider agroclimatic adaptability through interspecific hybridization (Ram et al. 2007; Basha and Sujatha 2009).

Understanding the genetic relationship between *Jatropha* species is prerequisite for successful interspecific hybridization. Sequence based molecular markers have been widely used for assessing interspecific relationship. The recent sequence based molecular phylogenetic tools based on spacers from nuclear genome and genes from plastid genome are very promising. The nuclear ribosomal (nr) internal transcribed spacer regions (ITS/5.8S) and chloroplast (cp) *matK* gene are the most common among these markers. The ITS region includes the conserved 5.8S subunit and fast evolving spacers ITS1 and ITS2 (Baldwin et al. 1995).The *matK* gene, encoding a splicing-associated maturase, is located in the group II intron of cpDNA *trnK* region (Neuhaus and Link 1987). Both of these regions have been widely employed for determination of phylogenetic relationship of plants at species level. However, very few studies have been undertaken for determining the phylogenetic relationship of agronomically important *Jatropha* species based on sequence based molecular phylogeny (Pamidimarri et al. 2009a, Basha and Sujatha 2009). Therefore, the present study was undertaken to infer phylogenetic relationship between *Jatropha* species using both nrDNA ITS and maternally inherited cpDNA based partial *matK* gene as markers.

6.2 Materials and methods

6.2.1 Collection of plant material and genomic DNA extraction

Jatropha species were collected from Assam (*J. podagrica*), Meghalaya (*J. curcas*) and Tripura (*J. gossypifolia*). The non toxic variety of *J. curcas* was obtained from Mexico. Genomic DNA was isolated from 500 mg of fresh and juvenile leaf samples of *J. podagrica*, *J. gossypifolia*, *J. curcas* (toxic) and *J. curcas* (non toxic) respectively following the standard CTAB method with minor modifications (Doyle and Doyle 1990). The DNA quality and concentration were estimated by spectrophotometric analysis as well as by visual comparison with known concentration of ladder in ethidium bromide stained 1% agarose gel. The extracted DNA was diluted to working concentration of 50 ng/ µl for ITS and *matK* based PCR analysis.

6.2.2 PCR amplification of ITS and *matK* region

6.2.2.1 ITS analysis

The nuclear rDNA ITS region was amplified by using the primers ITS1 F (5′-AGGATCATTGTCGAAACCT -3′) and ITS4 R (5′-GACGCTTCTCCAGACTACAA-3′). PCR amplifications were carried out in a 25 µl reaction mixture volume containing 50 ng of template DNA, 2.5 µl of reaction buffer, 100 µM of dNTP mix, l µM of forward and reverse primers and 1.0 U of Taq DNA polymerase. The PCR reactions were performed as follows: initial denaturation at 94°C for 4 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C, 2 min elongation at 72°C and final extension of 10 min at 72°C. PCR amplifications were carried out using a thermocycler (Applied Biosystems 2720).

6.2.2.2 *matK* analysis

The *matK* region was amplified by using the primer combinations *matK* F (5′-CGATCTATTCATTCAATATTTC-3′) and *matK* R (5′-TCTAGCACACGAAAGTCGAAGT-3′). PCR was performed on 25 µl volumes containing 50 ng of template DNA, 2.5 µl of reaction buffer, 100 µM of dNTP mix, l µM of forward and reverse primers and 1.0 U of Taq DNA polymerase. The PCR reactions were performed as follows: initial denaturation at 94°C for 4 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 51°C, 2 min elongation at 72°C and final extension of 10 min at 72°C.

6.2.3 Purification and sequencing of amplicons

The ITS and *matK* amplification fragments were resolved in 1.5% agarose gel. The ethidium bromide stained gels were photographed under ultraviolet light in gel documentation system (Bio-Rad Laboratories). The PCR amplicons were purified from excess salts and primers using HiPurA PCR Product Purification Spin Kit (Himedia) according to manufacturer's instructions. The sequences of purified ITS and *matK* fragments of *J. podagrica*, *J. gossypifolia* and *J. curcas* (toxic and non toxic varieties) were determined by using both forward and reverse primers by automated DNA sequencer (SciGenom Labs, Kochi, India). The nucleotide variations acquired were confirmed by resequencing. The sequences obtained have been deposited in Genbank.

6.2.4 Multiple Sequence Alignment

Sequences of cpDNA *matK* regions were trimmed to ensure that sequences for all species had the same start and end points. The sequence datas of ITS and *matK* regions of *Jatropha* species were aligned using the online alignment program, MultAlin implementing the default parameters [5]. The aligned sequences were compared for detection of indels and substitutions among *Jatropha* species and cultivars.

6.2.5 Data analysis

Transition/transversion ratios (R) were calculated using the formula $R= [A\text{x}G\text{x}k1+T\text{x}C\text{x}k2]/[(A+G)\text{x}(T+C)]$ (where, $k1$ and $k2$ are transition/transversion rate ratios for purines and pyrimidines respectively) with Molecular Evolutionary Genetics Analysis (MEGA) software version 5.2.(Tamura et al. 2011). Basic statistics like nucleotide compositions, pairwise genetic distances between *Jatropha* species and overall genetic distance were also calculated.

6.2.6 Phylogenetic analysis

For comparative analysis of the evolutionary relationship of ITS and *matK* regions in different *Jatropha* species, unrooted dendrogram based on Maximum Parsimony (MP) Method was constructed using MEGA implementing SPR (Subtree Pruning Regrafting) algorithm with the close-neighbor-interchange heuristic search. For this analysis, all molecular characters were assessed as equally weighted and unordered; furthermore, the alignment gaps were treated as missing data. Bootstrap analysis (1,000 replications) was executed for assessment of robustness of the phylogenetic analysis.

6.3 Results and discussion

6.3.1 nrDNA ITS region length and G+C content

The nrDNA ITS region of *J. podagrica*, *J. gossypifolia* and *J. curcas* (toxic and non-toxic varieties) were amplified with primers ITS1 F and ITS4 R. The amplification profile of nrDNA ITS region of *Jatropha* species has been depicted in Fig. 6.1. The nrDNA ITS sequences in this study included four regions: (1) ITS1 partial sequence, (2) 5.8S rDNA (3) ITS2 and (4) 25S rDNA partial sequence. The sequence data showed size variation of 790 bp (*J. podagrica*) to 793 bp (*J. gossypifolia*) in nrDNA ITS region (Table 6.1). The G+C content varied between 57.8 to 62.4%. The boundaries of ITS1, 5.8S rDNA, ITS2 and 25 rDNA were determined by comparing the sequences obtained with sequences from GenBank database (GenBank: AM774639.1). The length of ITS1, and 25SrDNA was constant in *Jatropha* species under investigation. ITS2 region varied from 195 bp (*J. podagrica*) to 197 bp (*J. gossypifolia*). A single base pair difference in the size of 5.8S region of *J. podagrica* (163 bp) was observed when compared with other *Jatropha* species (164 bp). The variation in G + C contents were 63.2 to 65.8% for ITS1, 48.5 to 56.1% for 5.8S rDNA, 60.5 to 68.4% for ITS2 and 55.2 to 55.8% for 25S rDNA region.

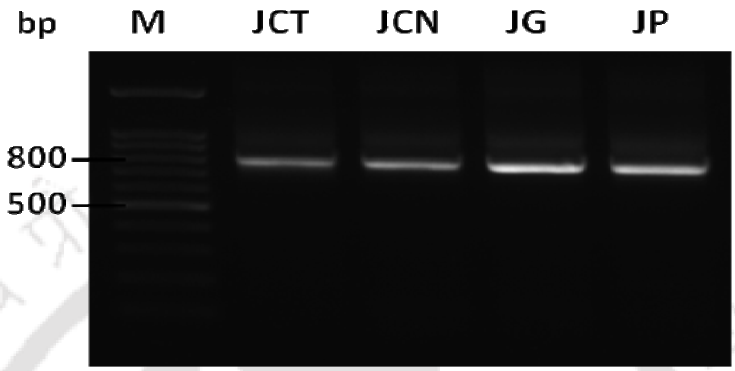


Fig.6.1 PCR amplification profile of *Jatropha* species using ITS1 F and ITS 4R primers. M = Marker, JCT = *J. curcas* (toxic variety), JCN = *J. curcas* (non toxic variety), JG = *J. gossypifolia*, JP = *J. podagrica*

Table 6.1 - ITS1, 5.8S, ITS2 and 25S length and G+C % content for *Jatropha* species

Species Name	**ITS**		**ITS1 (partial)**		**5.8S**		**ITS2**		**25S (partial)**	
	Length (bp)	**G+C (%)**	**Length (bp)**	**G+C (%)**	**Length (bp)**	**G+C (%)**	**Length (bp)**	**G+C (%)**	**Length (bp)**	**G+C (%)**
J. curcas (Toxic)	792	57.8	269	65.8	164	56.1	196	68.4	163	55.8
J. curcas (Non Toxic)	792	61.7	269	65.4	164	56.1	196	68.4	163	55.8
J. gossypifolia	793	62.4	269	64.7	164	56.1	197	67.5	163	55.2
J. podagrica	790	62.2	269	63.2	163	48.5	195	60.5	163	55.2

6.3.2 Multiple Sequence Alignment of nrDNA ITS dataset

The nrDNA ITS sequences of *Jatropha* species were aligned. The aligned datasets showed variations in 111 positions, 54 in ITS1, 16 in 5.8S, 38 in ITS2 and 3 in 25S region (Table 6.2, Fig. 6.2). Variations in ITS1-5.8S-ITS2-25S region included transition, transversion, insertion and deletion events. Comparison of the nrDNA ITS region of *Jatropha* species revealed 108 substitution events. It was observed that transition was higher than transversion, insertion and deletion (69% of total variable sites). Furthermore, transition from C to T was more frequent. The transition/ transversion bias (R) of ITS region was 2.68. The result obtained was in concordance with previous studies which have also reported that transitional substitutions occur at higher frequencies than transversional substitutions and are more probable in DNA sequences (Yang and Yoder 1999; Pamidimarri et al. 2009a). Multiple sequence alignment revealed three insertion/deletion events in ITS region. The insertions and deletions were single-base. No insertion/deletion events were found in ITS1 and 25S regions.

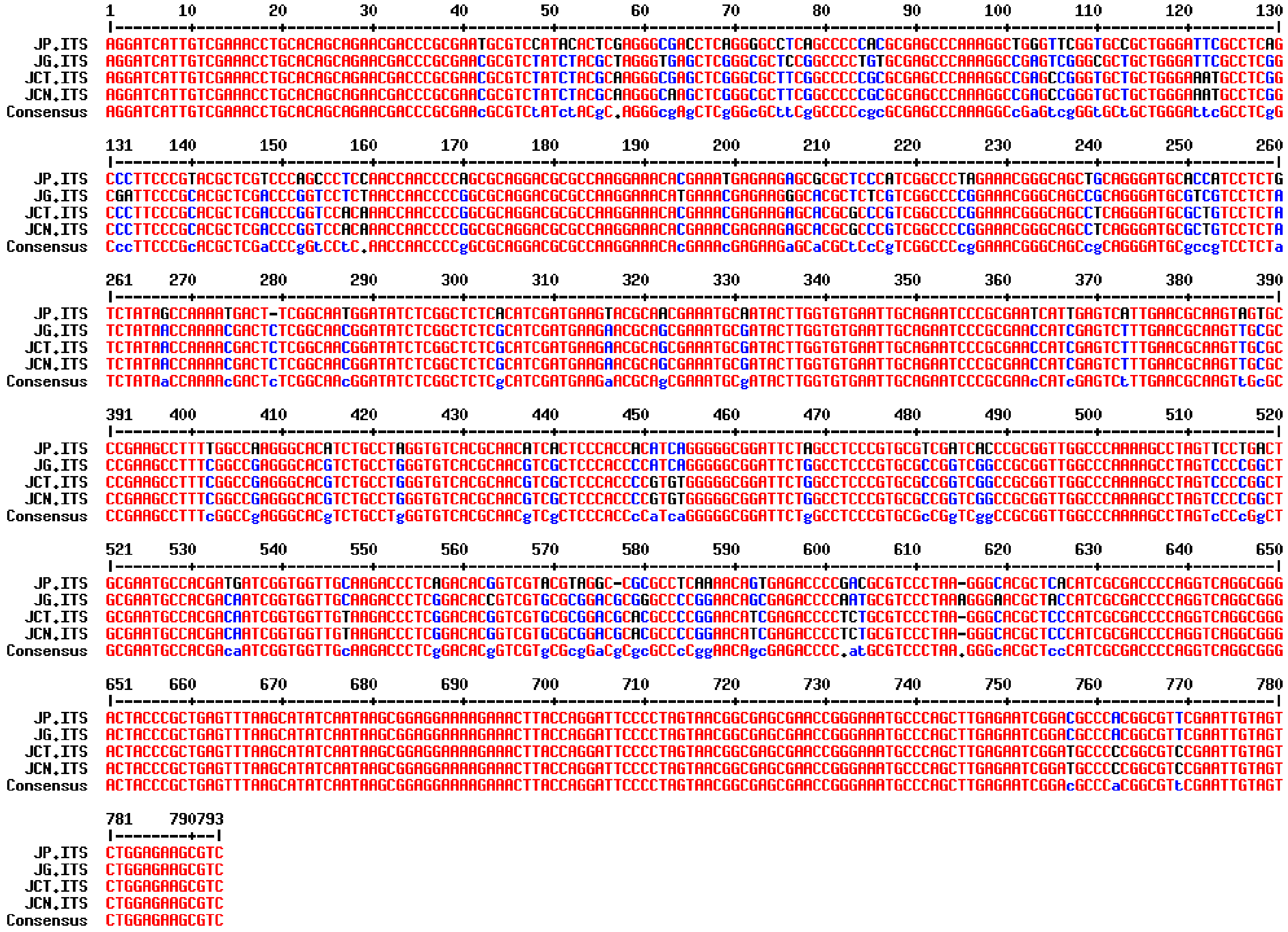
Table 6.2 – Summary of sequence analysis of ITS region in *Jatropha* species

Region	Variable sites	Substitution	Transition (Ti)	Transversion (Tv)	Ti+Tv	Indel	Insertion	Deletion
ITS1	54	54	39	14	1	-	-	-
5.8S	16	15	12	3	-	1	-	1
ITS2	38	36	24	12	-	2	1	1
25S	3	3	2	1		-	-	-
nrDNA ITS	**111**	**108**	**77**	**30**	**1**	**3**	**1**	**2**

The pairwise genetic distance and overall mean distance between *Jatropha* species were calculated using gamma distributed Kimura's two-parameter model with MEGA software version 5.2.(Tamura et al. 2011) (Table 6.3). Pairwise distance within the group varied from 0.001 to 0.139. It was observed that maximum distance (0.139) exists between *J. podagrica* and *J. curcas* (non toxic). On the other hand, *J. curcas* (toxic) and *J. curcas* (non toxic) exhibited minimum genetic distance (0.001). The average genetic distance between *Jatropha* species was 0.088.

Table 6.3 – ITS region based pairwise genetic distance and overall mean distance of *Jatropha* species

Organism	*J. podagrica*	*J. gossypifolia*	*J. curcas* (toxic)	Overall mean distance
J. gossypifolia	0.139			0.088±0.009
***J. curcas* (toxic)**	0.143	0.049		
***J. curcas* (non toxic)**	0.145	0.050	0.001	



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          1       10        20        30        40        50        60        70        80        90       100       110       120       130
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.ITS AGGATCATTGTCGAAACCTGCACAGCAGAACGACCCGCGAATGCGTCCATACACTCGAGGGCGACCTCAGGGGCCTCAGCCCCCACGCGAGCCCAAAGGCTGGGTTCGGTGCCGCTGGGATTCGCCTCAG
   JG.ITS AGGATCATTGTCGAAACCTGCACAGCAGAACGACCCGCGAACGCGTCTATCTACGCTAGGGTGAGCTCGGGCGCTCCGGCCCCTGTGCGAGCCCAAAGGCCGAGTCGGGCGCTGCTGGGATTCGCCTCGG
  JCT.ITS AGGATCATTGTCGAAACCTGCACAGCAGAACGACCCGCGAACGCGTCTATCTACGCAAGGGCGAGCTCGGGCGCTTCGGCCCCCGCGCGAGCCCAAAGGCCGAGCCGGGTGCTGCTGGGAAATGCCTCGG
  JCN.ITS AGGATCATTGTCGAAACCTGCACAGCAGAACGACCCGCGAACGCGTCTATCTACGCAAGGGCAAGCTCGGGCGCTTCGGCCCCCGCGCGAGCCCAAAGGCCGAGCCGGGTGCTGCTGGGAAATGCCTCGG
Consensus AGGATCATTGTCGAAACCTGCACAGCAGAACGACCCGCGAAcGCGTCtATctACgC.AGGGcgAgCTCgGGcGCttCgGCCCCcgcGCGAGCCCAAAGGCcGaGtcgGGtGCtGCTGGGAttcGCCTCgG

          131    140       150       160       170       180       190       200       210       220       230       240       250       260
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.ITS CCCTTCCCGTACGCTCGTCCCAGCCCTCCAACCAACCCCAGCGCAGGACGCGCCAAGGAAACACGAAATGAGAAGAGCGCGCTCCCATCGGCCCTAGAAACGGGCAGCTGCAGGGATGCACCATCCTCTG
   JG.ITS CGATTCCCGCACGCTCGACCCGGTCCTCTAACCAACCCCGGCGCAGGACGCGCCAAGGAAACATGAAACGAGAAGGGCACGCTCTCGTCGGCCCCGGAAACGGGCAGCCGCAGGGATGCGTCGTCCTCTA
  JCT.ITS CCCTTCCCGCACGCTCGACCCGGTCCACAAACCAACCCCGGCGCAGGACGCGCCAAGGAAACACGAAACGAGAAGAGCACGCGCCCGTCGGCCCCGGAAACGGGCAGCCTCAGGGATGCGCTGTCCTCTA
  JCN.ITS CCCTTCCCGCACGCTCGACCCGGTCCACAAACCAACCCCGGCGCAGGACGCGCCAAGGAAACACGAAACGAGAAGAGCACGCGCCCGTCGGCCCCGGAAACGGGCAGCCTCAGGGATGCGCTGTCCTCTA
Consensus CccTTCCCGcACGCTCGaCCCgGtCCtC.AACCAACCCCgGCGCAGGACGCGCCAAGGAAACAcGAAAcGAGAAGaGCaCGCtCcCgTCGGCCCcgGAAACGGGCAGCcgCAGGGATGCgccgTCCTCTa

          261    270       280       290       300       310       320       330       340       350       360       370       380       390
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.ITS TCTATAGCCAAAATGACT-TCGGCAATGGATATCTCGGCTCTCACATCGATGAAGTACGCAACGAAATGCAATACTTGGTGTGAATTGCAGAATCCCGCGAATCATTGAGTCATTGAACGCAAGTAGTGC
   JG.ITS TCTATAACCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAAGTTGCGC
  JCT.ITS TCTATAACCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAAGTTGCGC
  JCN.ITS TCTATAACCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAAGTTGCGC
Consensus TCTATAaCCAAAAcGACTcTCGGCAAcGGATATCTCGGCTCTCgCATCGATGAAGaACGCAgCGAAATGCgATACTTGGTGTGAATTGCAGAATCCCGCGAAcCATcGAGTCtTTGAACGCAAGTtGcGC

          391    400       410       420       430       440       450       460       470       480       490       500       510       520
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.ITS CCGAAGCCTTTTGGCCAAGGGCACATCTGCCTAGGTGTCACGCAACATCACTCCCACCACATCAGGGGGCGGATTCTAGCCTCCCGTGCGTCGATCACCCGCGGTTGGCCCAAAAGCCTAGTTCCTGACT
   JG.ITS CCGAAGCCTTTCGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAACGTCGCTCCCACCCCATCAGGGGGCGGATTCTGGCCTCCCGTGCGCCGGTCGGCCGCGGTTGGCCCAAAAGCCTAGTCCCCGGCT
  JCT.ITS CCGAAGCCTTTCGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAACGTCGCTCCCACCCCGTGTGGGGGCGGATTCTGGCCTCCCGTGCGCCGGTCGGCCGCGGTTGGCCCAAAAGCCTAGTCCCCGGCT
  JCN.ITS CCGAAGCCTTTCGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAACGTCGCTCCCACCCCGTGTGGGGGCGGATTCTGGCCTCCCGTGCGCCGGTCGGCCGCGGTTGGCCCAAAAGCCTAGTCCCCGGCT
Consensus CCGAAGCCTTTcGGCCgAGGGCACgTCTGCCTgGGTGTCACGCAACgTCgCTCCCACCcCaTcaGGGGGCGGATTCTgGCCTCCCGTGCGcCGgTCggCCGCGGTTGGCCCAAAAGCCTAGTcCCcGgCT

          521    530       540       550       560       570       580       590       600       610       620       630       640       650
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.ITS GCGAATGCCACGATGATCGGTGGTTGCAAGACCCTCAGACACGGTCGTACGTAGGC-CGCGCCTCAAAACAGTGAGACCCCGACGCGTCCCTAA-GGGCACGCTCACATCGCGACCCCAGGTCAGGCGGG
   JG.ITS GCGAATGCCACGACAATCGGTGGTTGCAAGACCCTCGGACACCGTCGTGCGCGGACGCGGGCCCCGGAACAGCGAGACCCCAATGCGTCCCTAAAGGGAACGCTACCATCGCGACCCCAGGTCAGGCGGG
  JCT.ITS GCGAATGCCACGACAATCGGTGGTTGTAAGACCCTCGGACACGGTCGTGCGCGGACGCACGCCCCGGAACATCGAGACCCCTCTGCGTCCCTAA-GGGCACGCTCCCATCGCGACCCCAGGTCAGGCGGG
  JCN.ITS GCGAATGCCACGACAATCGGTGGTTGTAAGACCCTCGGACACGGTCGTGCGCGGACGCACGCCCCGGAACATCGAGACCCCTCTGCGTCCCTAA-GGGCACGCTCCCATCGCGACCCCAGGTCAGGCGGG
Consensus GCGAATGCCACGAcaATCGGTGGTTGcAAGACCCTCgGACACgGTCGTgCGcgGaCgCgcGCCcCggAACAgcGAGACCCC.atGCGTCCCTAA.GGGcACGCTccCATCGCGACCCCAGGTCAGGCGGG

          651    660       670       680       690       700       710       720       730       740       750       760       770       780
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.ITS ACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTTACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAATGCCCAGCTTGAGAATCGGACGCCCACGGCGTTCGAATTGTAGT
   JG.ITS ACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTTACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAATGCCCAGCTTGAGAATCGGACGCCCACGGCGTTCGAATTGTAGT
  JCT.ITS ACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTTACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAATGCCCAGCTTGAGAATCGGATGCCCCCGGCGTCCGAATTGTAGT
  JCN.ITS ACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTTACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAATGCCCAGCTTGAGAATCGGATGCCCCCGGCGTCCGAATTGTAGT
Consensus ACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTTACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAATGCCCAGCTTGAGAATCGGAcGCCCaCGGCGTtCGAATTGTAGT

          781    790793
          |--------+--|
   JP.ITS CTGGAGAAGCGTC
   JG.ITS CTGGAGAAGCGTC
  JCT.ITS CTGGAGAAGCGTC
  JCN.ITS CTGGAGAAGCGTC
Consensus CTGGAGAAGCGTC
```

Fig.6.2 nrDNA ITS region based Multiple Sequence Alignment of *Jatropha* species

6.3.3 Phylogenetic analysis of ITS sequences

The ITS datasets of *Jatropha* species were analyzed through Maximum Parsimony Analysis. A single tree was generated (Fig. 6.3). *J. curcas* and *J. podagrica* maintained maximum distance in the nrDNA ITS sequence based phylogram. On the other hand, the toxic and non toxic varieties of *J. curcas* were clustered together. *J. podagrica* showed maximum similarity to the consensus sequence. The phylogenetic relationship obtained from Maximum Parsimony Analysis well supported the pairwise genetic distance estimated between *Jatropha* species using Kimura's two-parameter model (Table 6.3).

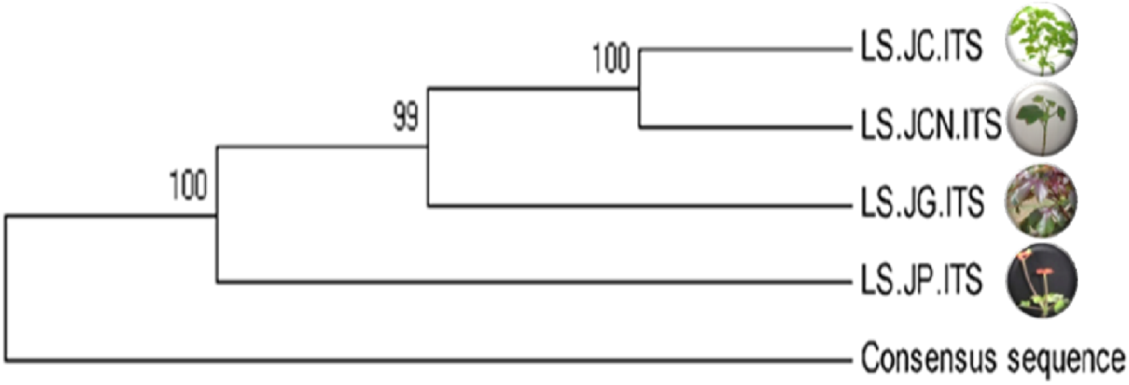


Fig.6.3 nrDNA ITS region based Maximum Parsimony Tree of *Jatropha* species

6.3.4 cpDNA *matK* region length and G+C content

The cpDNA *matK* region of *J. podagrica*, *J. gossypifolia* and *J. curcas* (toxic and non-toxic varieties) were amplified with primers *matK* F and *matK* R. The amplification profile of cpDNA *matK* region of *Jatropha* species has been showed in Fig. 6.4. The sequence data depicted that the length of *matK* region under investigation is constant across *Jatropha* species (Table 6.4). The variations in G + C contents were 31.2% (*J. podagrica*) to 31.4% (*J. gossypifolia*). In contrast to ITS region, markedly low G+C content characterized the *matK* region of *Jatropha* species.

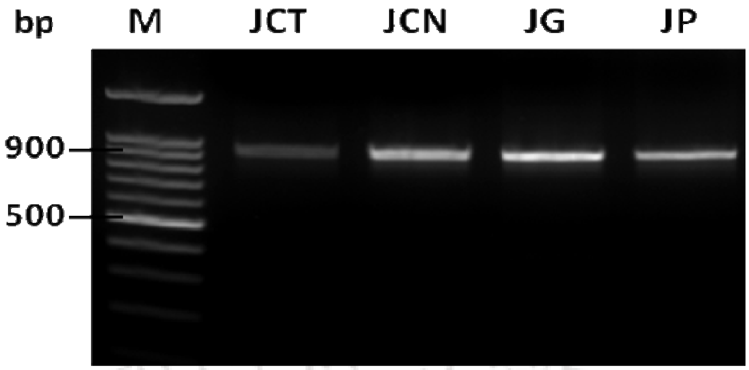


Fig.6.4 PCR amplification profile of *Jatropha* species using *matK* F and *matK* R primers. M = Marker, JCT = *J. curcas* (toxic variety), JCN = *J. curcas* (non toxic variety), JG = *J. gossypifolia*, JP = *J. podagrica*

Table 6.4 – length of partial *matK* gene and G+C % content for *Jatropha* species

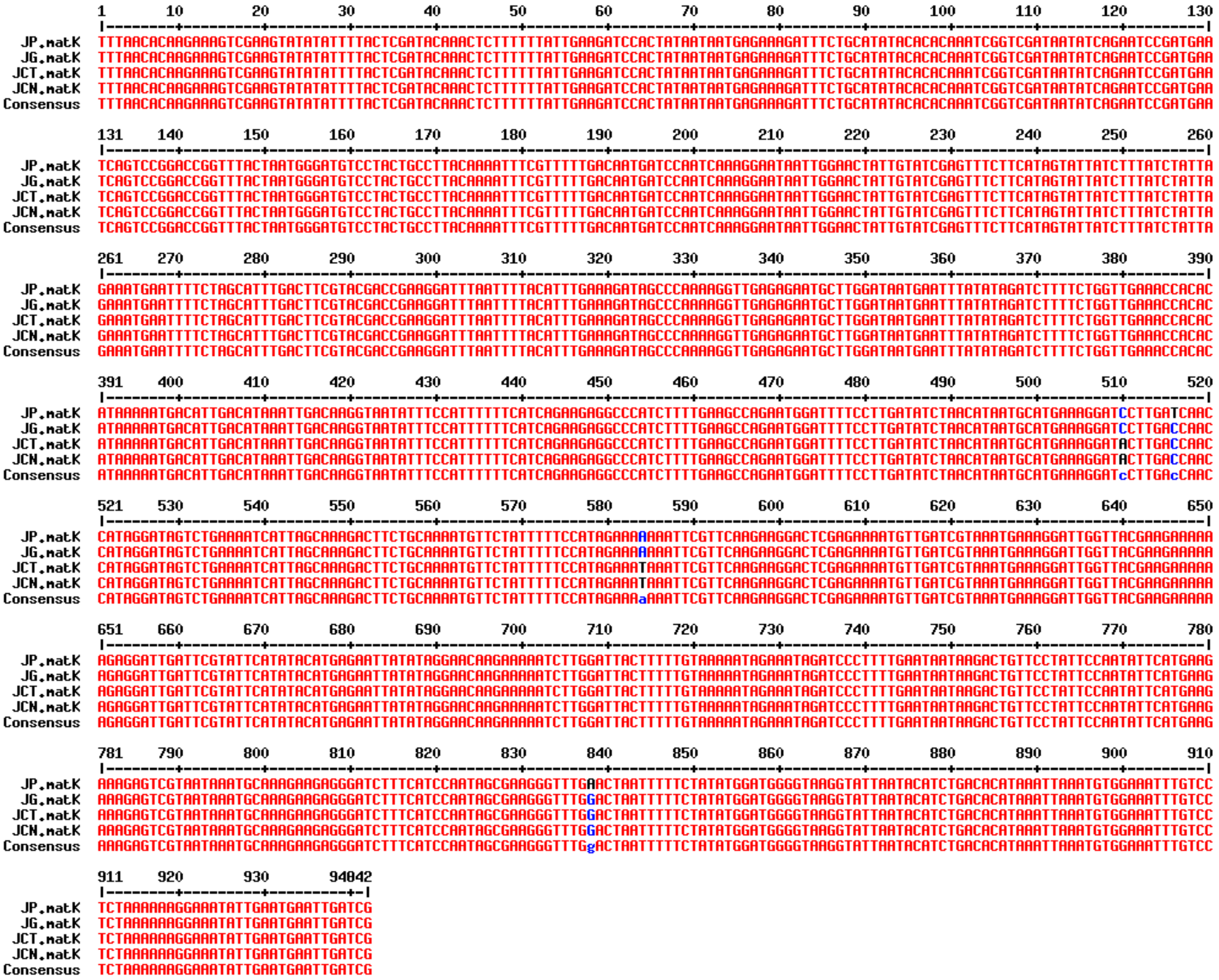
Species Name	***matK***	
	Length (bp)	**G+C (%)**
J. curcas (Toxic)	942	31.3
J. curcas (Non Toxic)	942	31.3
J. gossypifolia	942	31.4
J. podagrica	942	31.2

6.3.5 Multiple Sequence Alignment of cpDNA *matK* dataset

The cpDNA *matK* sequences of *Jatropha* species were aligned (Fig. 6.5). The aligned datasets showed substitution in 4 positions. Transitions were observed in 516 bp and 838 bp. Transversions were observed in 510 bp and 584 bp. In contrast to nrDNA ITS region, no indels were observed in cpDNA *matK* sequences of *Jatropha* species (Table 6.5).

Table 6.5 – Summary of sequence analysis of matK region in *Jatropha* species

Region	Variable sites	Substitution	Transition (Ti)	Transversion (Tv)	Indel
matK	4	4	2	2	-



```
           1       10        20        30        40        50        60        70        80        90       100       110       120       130
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.matK TTTAACACAAGAAAGTCGAAGTATATATTTTACTCGATACAAACTCTTTTTTATTGAAGATCCACTATAATAATGAGAAAGATTTCTGCATATACACACAAATCGGTCGATAATATCAGAATCCGATGAA
   JG.matK TTTAACACAAGAAAGTCGAAGTATATATTTTACTCGATACAAACTCTTTTTTATTGAAGATCCACTATAATAATGAGAAAGATTTCTGCATATACACACAAATCGGTCGATAATATCAGAATCCGATGAA
  JCT.matK TTTAACACAAGAAAGTCGAAGTATATATTTTACTCGATACAAACTCTTTTTTATTGAAGATCCACTATAATAATGAGAAAGATTTCTGCATATACACACAAATCGGTCGATAATATCAGAATCCGATGAA
  JCN.matK TTTAACACAAGAAAGTCGAAGTATATATTTTACTCGATACAAACTCTTTTTTATTGAAGATCCACTATAATAATGAGAAAGATTTCTGCATATACACACAAATCGGTCGATAATATCAGAATCCGATGAA
 Consensus TTTAACACAAGAAAGTCGAAGTATATATTTTACTCGATACAAACTCTTTTTTATTGAAGATCCACTATAATAATGAGAAAGATTTCTGCATATACACACAAATCGGTCGATAATATCAGAATCCGATGAA

           131    140       150       160       170       180       190       200       210       220       230       240       250       260
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.matK TCAGTCCGGACCGGTTTACTAATGGGATGTCCTACTGCCTTACAAAATTTCGTTTTTGACAATGATCCAATCAAAGGAATAATTGGAACTATTGTATCGAGTTTCTTCATAGTATTATCTTTATCTATTA
   JG.matK TCAGTCCGGACCGGTTTACTAATGGGATGTCCTACTGCCTTACAAAATTTCGTTTTTGACAATGATCCAATCAAAGGAATAATTGGAACTATTGTATCGAGTTTCTTCATAGTATTATCTTTATCTATTA
  JCT.matK TCAGTCCGGACCGGTTTACTAATGGGATGTCCTACTGCCTTACAAAATTTCGTTTTTGACAATGATCCAATCAAAGGAATAATTGGAACTATTGTATCGAGTTTCTTCATAGTATTATCTTTATCTATTA
  JCN.matK TCAGTCCGGACCGGTTTACTAATGGGATGTCCTACTGCCTTACAAAATTTCGTTTTTGACAATGATCCAATCAAAGGAATAATTGGAACTATTGTATCGAGTTTCTTCATAGTATTATCTTTATCTATTA
 Consensus TCAGTCCGGACCGGTTTACTAATGGGATGTCCTACTGCCTTACAAAATTTCGTTTTTGACAATGATCCAATCAAAGGAATAATTGGAACTATTGTATCGAGTTTCTTCATAGTATTATCTTTATCTATTA

           261    270       280       290       300       310       320       330       340       350       360       370       380       390
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.matK GAAATGAATTTTCTAGCATTTGACTTCGTACGACCGAAGGATTTAATTTTACATTTGAAAGATAGCCCAAAAGGTTGAGAGAATGCTTGGATAATGAATTTATATAGATCTTTTCTGGTTGAAACCACAC
   JG.matK GAAATGAATTTTCTAGCATTTGACTTCGTACGACCGAAGGATTTAATTTTACATTTGAAAGATAGCCCAAAAGGTTGAGAGAATGCTTGGATAATGAATTTATATAGATCTTTTCTGGTTGAAACCACAC
  JCT.matK GAAATGAATTTTCTAGCATTTGACTTCGTACGACCGAAGGATTTAATTTTACATTTGAAAGATAGCCCAAAAGGTTGAGAGAATGCTTGGATAATGAATTTATATAGATCTTTTCTGGTTGAAACCACAC
  JCN.matK GAAATGAATTTTCTAGCATTTGACTTCGTACGACCGAAGGATTTAATTTTACATTTGAAAGATAGCCCAAAAGGTTGAGAGAATGCTTGGATAATGAATTTATATAGATCTTTTCTGGTTGAAACCACAC
 Consensus GAAATGAATTTTCTAGCATTTGACTTCGTACGACCGAAGGATTTAATTTTACATTTGAAAGATAGCCCAAAAGGTTGAGAGAATGCTTGGATAATGAATTTATATAGATCTTTTCTGGTTGAAACCACAC

           391    400       410       420       430       440       450       460       470       480       490       500       510       520
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.matK ATAAAAATGACATTGACATAAATTGACAAGGTAATATTTCCATTTTTTCATCAGAAGAGGCCCATCTTTTGAAGCCAGAATGGATTTTCCTTGATATCTAACATAATGCATGAAAGGATCCTTGATCAAC
   JG.matK ATAAAAATGACATTGACATAAATTGACAAGGTAATATTTCCATTTTTTCATCAGAAGAGGCCCATCTTTTGAAGCCAGAATGGATTTTCCTTGATATCTAACATAATGCATGAAAGGATCCTTGACCAAC
  JCT.matK ATAAAAATGACATTGACATAAATTGACAAGGTAATATTTCCATTTTTTCATCAGAAGAGGCCCATCTTTTGAAGCCAGAATGGATTTTCCTTGATATCTAACATAATGCATGAAAGGATACTTGACCAAC
  JCN.matK ATAAAAATGACATTGACATAAATTGACAAGGTAATATTTCCATTTTTTCATCAGAAGAGGCCCATCTTTTGAAGCCAGAATGGATTTTCCTTGATATCTAACATAATGCATGAAAGGATACTTGACCAAC
 Consensus ATAAAAATGACATTGACATAAATTGACAAGGTAATATTTCCATTTTTTCATCAGAAGAGGCCCATCTTTTGAAGCCAGAATGGATTTTCCTTGATATCTAACATAATGCATGAAAGGATcCTTGAcCAAC

           521    530       540       550       560       570       580       590       600       610       620       630       640       650
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.matK CATAGGATAGTCTGAAAATCATTAGCAAAGACTTCTGCAAAATGTTCTATTTTTCCATAGAAAAAAATTCGTTCAAGAAGGACTCGAGAAAATGTTGATCGTAAATGAAAGGATTGGTTACGAAGAAAAA
   JG.matK CATAGGATAGTCTGAAAATCATTAGCAAAGACTTCTGCAAAATGTTCTATTTTTCCATAGAAAAAAATTCGTTCAAGAAGGACTCGAGAAAATGTTGATCGTAAATGAAAGGATTGGTTACGAAGAAAAA
  JCT.matK CATAGGATAGTCTGAAAATCATTAGCAAAGACTTCTGCAAAATGTTCTATTTTTCCATAGAAATAAATTCGTTCAAGAAGGACTCGAGAAAATGTTGATCGTAAATGAAAGGATTGGTTACGAAGAAAAA
  JCN.matK CATAGGATAGTCTGAAAATCATTAGCAAAGACTTCTGCAAAATGTTCTATTTTTCCATAGAAATAAATTCGTTCAAGAAGGACTCGAGAAAATGTTGATCGTAAATGAAAGGATTGGTTACGAAGAAAAA
 Consensus CATAGGATAGTCTGAAAATCATTAGCAAAGACTTCTGCAAAATGTTCTATTTTTCCATAGAAAaAAATTCGTTCAAGAAGGACTCGAGAAAATGTTGATCGTAAATGAAAGGATTGGTTACGAAGAAAAA

           651    660       670       680       690       700       710       720       730       740       750       760       770       780
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.matK AGAGGATTGATTCGTATTCATATACATGAGAATTATATAGGAACAAGAAAAATCTTGGATTACTTTTTGTAAAAATAGAAATAGATCCCTTTTGAATAATAAGACTGTTCCTATTCCAATATTCATGAAG
   JG.matK AGAGGATTGATTCGTATTCATATACATGAGAATTATATAGGAACAAGAAAAATCTTGGATTACTTTTTGTAAAAATAGAAATAGATCCCTTTTGAATAATAAGACTGTTCCTATTCCAATATTCATGAAG
  JCT.matK AGAGGATTGATTCGTATTCATATACATGAGAATTATATAGGAACAAGAAAAATCTTGGATTACTTTTTGTAAAAATAGAAATAGATCCCTTTTGAATAATAAGACTGTTCCTATTCCAATATTCATGAAG
  JCN.matK AGAGGATTGATTCGTATTCATATACATGAGAATTATATAGGAACAAGAAAAATCTTGGATTACTTTTTGTAAAAATAGAAATAGATCCCTTTTGAATAATAAGACTGTTCCTATTCCAATATTCATGAAG
 Consensus AGAGGATTGATTCGTATTCATATACATGAGAATTATATAGGAACAAGAAAAATCTTGGATTACTTTTTGTAAAAATAGAAATAGATCCCTTTTGAATAATAAGACTGTTCCTATTCCAATATTCATGAAG

           781    790       800       810       820       830       840       850       860       870       880       890       900       910
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   JP.matK AAAGAGTCGTAATAAATGCAAAGAAGAGGGATCTTTCATCCAATAGCGAAGGGTTTGAACTAATTTTTCTATATGGATGGGGTAAGGTATTAATACATCTGACACATAAATTAAATGTGGAAATTTGTCC
   JG.matK AAAGAGTCGTAATAAATGCAAAGAAGAGGGATCTTTCATCCAATAGCGAAGGGTTTGGACTAATTTTTCTATATGGATGGGGTAAGGTATTAATACATCTGACACATAAATTAAATGTGGAAATTTGTCC
  JCT.matK AAAGAGTCGTAATAAATGCAAAGAAGAGGGATCTTTCATCCAATAGCGAAGGGTTTGGACTAATTTTTCTATATGGATGGGGTAAGGTATTAATACATCTGACACATAAATTAAATGTGGAAATTTGTCC
  JCN.matK AAAGAGTCGTAATAAATGCAAAGAAGAGGGATCTTTCATCCAATAGCGAAGGGTTTGGACTAATTTTTCTATATGGATGGGGTAAGGTATTAATACATCTGACACATAAATTAAATGTGGAAATTTGTCC
 Consensus AAAGAGTCGTAATAAATGCAAAGAAGAGGGATCTTTCATCCAATAGCGAAGGGTTTGgACTAATTTTTCTATATGGATGGGGTAAGGTATTAATACATCTGACACATAAATTAAATGTGGAAATTTGTCC

           911    920       930      94042
           |--------+---------+---------+-|
   JP.matK TCTAAAAAAGGAAATATTGAATGAATTGATCG
   JG.matK TCTAAAAAAGGAAATATTGAATGAATTGATCG
  JCT.matK TCTAAAAAAGGAAATATTGAATGAATTGATCG
  JCN.matK TCTAAAAAAGGAAATATTGAATGAATTGATCG
 Consensus TCTAAAAAAGGAAATATTGAATGAATTGATCG
```

Fig.6.5 cpDNA *matK* region based Multiple Sequence Alignment of *Jatropha* species

The *matK* region based pairwise genetic distance and overall mean distance between *Jatropha* species were estimated using the Kimura two-parameter model (Table 6.6). The Kimura two-parameter distance varied from 0 to 0.004. The overall mean distance was 0.002. It was found that maximum interspecific distance (0.004) exists between *J. podagrica* and *J. curcas*. On the other hand, no intraspecific distance was observed between *J. curcas* toxic and non toxic varieties.

Table 6.6 – *matK* region based pairwise genetic distance and overall mean distance of *Jatropha* species

Organism	*J. podagrica*	*J. gossypifolia*	*J. curcas* (toxic)	Overall mean distance
J. gossypifolia	0.002			0.002±0.001
***J. curcas* (toxic)**	0.004	0.002		
***J. curcas* (non toxic)**	0.004	0.002	0.000	

6.3.6 Phylogenetic analysis of *matK* sequences

The *matK* region based maximum parsimony analysis of *Jatropha* species resulted in a single parsimonious tree (Fig. 6.6). *J. curcas* toxic and non toxic varieties formed together a single clade. *J. curcas* and *J. podagrica* maintained maximum distance in the parsimony tree. This result is also supported by Kimura two-parameter model based distance analysis. It was also observed that comparatively more conserved chloroplast DNA *matK* based phylogram have high consistency with less conserved nuclear DNA ITS based phylogram of *Jatropha* species.

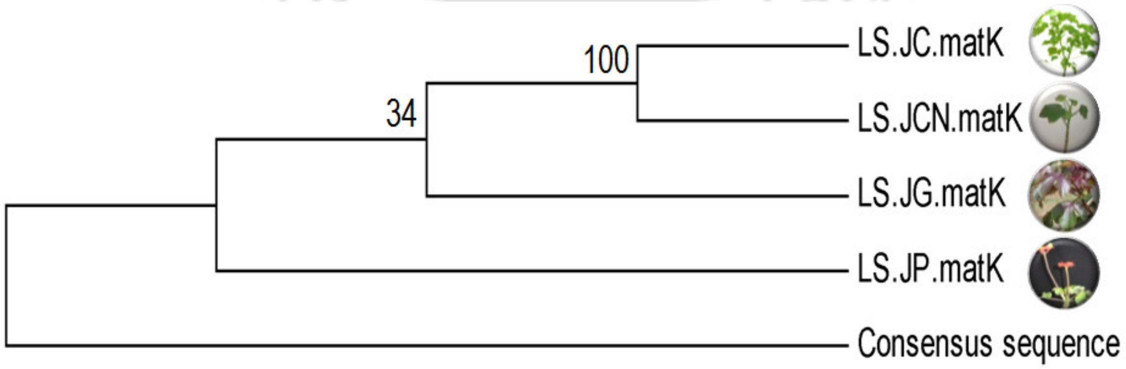


Fig.6.6 cpDNA *matK* region based Maximum Parsimony Tree of *Jatropha* species

6.4 Conclusion

In this study the interspecific relationship of *J. curcas* with the sister species *J. podagrica* and *J. gossypifolia* has been delineated using nrDNA ITS markers and cpDNA *matK* markers. From the present study, it was concluded that nrDNA ITS region in *Jatropha* is comparatively more informative than cpDNA *matK* region. The estimation of pairwise genetic distance and maximum parsimony analysis revealed that for both ITS and *matk* region, maximum genetic distance exists between *J. curcas* and *J. podagrica.* From the study, it was also inferred that both ITS and matK region are highly conserved between *J. curcas* toxic and non toxic varieties. The variable sites obtained in ITS and *matK* region of *Jatropha* species can later be used as effectives markers for species identification. In future, further studies of nrDNA ITS and cpDNA *matK* region of *Jatropha* involving more number of species may provide important information about the molecular evolution of this species.

Chapter 7

Concluding remarks

7.1 Significance and salient features of the study

J. curcas has attracted a great deal of global attention as a drought resistant oil-rich biodiesel plant. The fact that *J. curcas* can survive in non-arable lands with no competition with food crops has resulted in large scale *J. curcas* plantation in Indian subcontinent which has large tracts of wastelands suitable for cultivation of this plant. However, the net financial gain from *J. curcas* is low as the plant often suffers from seed toxicity, poor and inconsistent seed yield and morphological characters. Development of improved varieties of *J. curcas* using genetic engineering and molecular breeding tools is required for commercial exploitation of the plant. The key for success of any tree improvement venture lies in the magnitude of existing phenotypic and genetic variability within the germplasm. However, presence of limited genetic diversity within the available *J. curcas* germplasm is of special concern for tree improvement programs. Thus germplasm evaluation programs involving systematic collection of *J. curcas* populations from wild habitat with subsequent characterization of morphological, genetic and biochemical variation within the wild populations is required for identification of superior plants.

The present study was carried out to comprehensively assess the morphological variations, molecular diversity and variation in phorbol ester content of *J. curcas* populations from North-East India. The genetic diversity within *Jatropha* species, commonly available in Noth-East India, was also assessed. The investigation was carried out in four distinct phases. In the first phase, twenty-nine *J. curcas* populations were collected from eco-geographically distinct regions of North-East India. Four populations from outside North-East were also incorporated in the study so that a detailed comparison can be made between North-East and outside North-East populations. The morphological trait based analysis of *J. curcas* populations revealed high variability of quantitative traits. The highest variation (%CV=23.19) was observed in M: F flower ratio while CS appeared as least variable trait (%CV=1.67). Based on morphometric trait values, the three populations from Assam (IITJC15, IITJC24 and IITJC28) and one population from Arunachal Pradesh (IITJC7) were found to be superior in comparison with the other populations. Cluster and principal component analyses depicted that the populations

IITJC19, IITJC21, IITJC22 and IITJC24 from Assam have maintained maximum inter-cluster distance from the rest of the populations. The analyses also depicted that no clear demarcation can be done between North-East and outside North-East populations on the basis of morphometric variability. Morphometric characterization of *J. curcas* populations leads to the identification of seven promising populations (IITJC7, IITJC15, IITJC19, IITJC21, IITJC22, IITJC24 and IITJC28) from North-East India. From the study of variance component and broad sense heritability, it was inferred that selection of elite plants on the basis of M: F ratio, 100SW and TSY ($h^2_{bs} > 75\%$) in tree improvement programs is likely to be more successful.

The second phase of investigation involved molecular characterization of thirty three *J. curcas* populations by employing random markers like RAPD and ISSR, and curcin gene specific markers. Significant polymorphism was observed with RAPD (67.71% of polymorphism) and ISSR (66.13% of polymorphism) however curcin specific primer pairs showed monomorphism across all *J. curcas* populations. From the results it was inferred that both the random markers were equally efficient in detecting polymorphism across *J. curcas* populations. From high values of Nei's gene diversity and Shannon's information index for North-East populations it was suggested that *J. curcas* plant from North-East harbors higher genetic variation. The grouping pattern in cluster and principal component analyses was also in accordance with the above fact. However, the number of outside North-East populations considered for diversity studies was less. It was found that IITJC5, IITJC6 and IITJC7 from Arunachal Pradesh, IITJC12 and IITJC13 from Meghalaya and IITJC14 from Tripura have maintained maximum genetic distance from all other populations. On the other hand, nesting of Manipur and Mizoram populations and some populations of Assam with outgroups in a single cluster suggested dispersion of *J. curcas* plant from outside North-East to some parts of North-East India. From the present study, seven *J. curcas* populations from Arunachal Pradesh (IITJC5, IITJC6 and IITJCC7), Meghalaya (IITJC12 and IITJC13), Tripura (IITJC14) and Assam (IITJC15) were identified as genetically diverse. In future, genetic diversity studies involving more number of *J. curcas* plants from outside North-East will help in clear depiction of extent of genetic diversity between North-East and outside North-East populations.

In the third phase, seed phorbol ester content of *J. curcas* populations, identified as promising in previous studies, was estimated. Presence of at least six phorbol ester factors in *J. curcas* was confirmed. Significant variation (1.02 mg/g to 2.75 mg/g) in phorbol ester concentration among *J. curcas* populations of North-East India was obtained. It has been previously reported that total phorbol ester content in toxic *J. curcas* seeds varies within the range of 0.87 mg/g to 3.32 mg/g (Makkar et al. 1997). By comparing with the previous reports, IITJC12 (1.02 mg/g of seed kernel), IITJC7 (1.15 mg/g of seed kernel) and IITJC15 (1.38 mg/g of seed kernel), having comparatively low seed phorbol ester content were identified as low phorbol ester containing populations.

The present investigations clearly revealed considerable phenotypic, molecular and phorbol ester-based biochemical diversity among *J. curcas* populations from North-East India. The seven populations, IITJC7, IITJC15, IITJC19, IITJC21, IITJC22, IITJC24 and IITJC28, emerged as superior on the basis of morphometric traits. On the other hand, the molecular diversity studies suggested IITJC5, IITJC6, IITJC7, IITJC12, IITJC13, IITJC14 and IITJC15 as promising populations having considerable genetic divergence. For correlating the phenotypic and molecular diversity studies, the molecular characterization results were coupled with morphometric data. Collation of morphometric and molecular variations elucidated that IITJC15 (Assam) having maximum total seed yield along with minimum male to female flower ratio and IITJC7 (Arunachal Pradesh) having highest 100 seed weight are both morphometrically promising and genetically distinct. Interestingly it was observed that IITJC7 and IITJC15 are also having low seed phorbol ester content. The promising *J. curcas* populations identified in this investigation in terms of yield attributing traits complementarily with genetic heterogeneity and seed phorbol ester content can be recommended as potential starting materials in tree breeding programs for the development of genetically diverse *Jatropha* genotypes with desirable agronomic and biochemical traits.

In the last phase of this study, genetic relationship of *Jatropha* species (*J. curcas*, *J. podagrica* and *J. gossypifolia*) commonly available in North-East India was determined using nuclear DNA specific ITS marker and chloroplast DNA specific *matK* marker. A total of 115 variable sites were detected in ITS and *matK* regions of *Jatropha* species. From the sequence data obtained from ITS and *matK* region, it was inferred that the ITS markers are more efficient in detecting indels and substitutions in *Jatropha* species. Maximum parimony tree based analysis elucidated the genetic relationship between *Jatropha* species. It was found that maximum genetic distance exists between *J. curcas* and *J. podagrica*.

The salient features of the present study are summarized below:

- Seven promising populations of *J. curcas* (IITJC7, IITJC15, IITJC19, IITJC21, IITJC22, IITJC24 and IITJC28) from Arunachal Pradesh and Assam were identified on the basis of morphometric traits.
- Molecular characterization of *J. curcas* germplasm helped in identification of seven genetically distinct populations (IITJC15, IITJC5, IITJC6, IITJC7, IITJC12, IITJC13 and IITJC14) from Assam and geographically isolated states of Arunachal Pradesh, Meghalaya and Tripura.
- Biochemical analyses of *J. curcas* seeds lead to the identification of three populations (IITJC7, IITJC12 and IITJC15) from North-East having low seed phorbol ester content.
- Complementation of morphometric, molecular and biochemical studies indicated IITJC7 and IITJC15 as genetically distinct populations having superior morphometric traits and low seed phorbol ester content.
- Nuclear DNA ITS region and Chloroplast DNA *matK* region based genetic relationship of *Jatropha* species was determined. Substitutions and Indels which can later be used as potential markers were detected.

This study will provide guidelines for successful exploitation of untapped *J. curcas* genetic resource of North-East India for genetic improvement of the plant.

7.2 Future prospects

Morphometric, molecular and biochemical characterization of economically important plants help in identification of elite plants which can later be used as starting material for tree improvement programs. To our knowledge, this is the first comprehensive report where in complementation with genetic diversity studies, agronomically important morphometric and biochemical traits of *J. curcas* populations from North-East India has also been estimated. The results obtained in our comprehensive study will provide framework for future *J. curcas* improvement ventures. The scope of the findings of the present investigation on future research has been discussed in details.

The six morphometric traits, plant height, canopy spread, collar diameter, male to female flower ratio, 100 seed weight and total seed yield, used in characterization of *J. curcas* germplasm from North-East, were efficient in screening the populations. From the morphometric study it was suggested that during future *J. curcas* evaluation programs, growth attributes like plant height, canopy spread and collar diameter can be used as initial screening indices of young trees. However, once the tree attains gestation period, selection of agronomically promising plants on the basis of male to female flower ratio, 100 seed weight and total seed yield is likely to be more effective. Use of *J. curcas* populations, having superior plant height and canopy spread (IITJC24), maximum collar diameter (IITJC28), maximum 100 seed weight (IITJC7) and highest total seed yield (IITJC15) as parents in tree breeding programs will help in development of trait specific *J. curcas* varieties in the next generation. Alongside, selection of parents from IITJC19, IITJC21, IITJC22 and IITJC24, will help in generation of *J. curcas* plants with greater genetic heterogeneity.

The primers used in this investigation for molecular characterization of *J. curcas* germplasm can later be used as primordial tools for identifying genetically distinct populations from wild or naturalized *J. curcas* populations. In future, the DNA fingerprinting profiles obtained in this study will be linked with morphometric or biochemical traits of *J. curcas* for development of trait specific Sequenced Characterized Amplified Region Markers which will facilitate Marker Assisted Selection in *J. curcas*. Furthermore, the seven genetically diverse *J. curcas* populations, IITJC5, IITJC6 and IITJC7 from Arunachal Pradesh, IITJC12 and IITJC13 from Meghalaya, IITJC14 from Tripura and IITJC15 from Assam, identified in this study are valuable resources for broadening the genetic base of *J. curcas* germplasm in India.

Phorbol ester, a cocarcinogen, found in *J. curcas* seeds is the major impediment behind successful utilization of left over seed cakes as animal feed after oil-extraction. Even though, presence non-toxic variety of *J. curcas* has been reported only from Mexico, however, introduction of non-toxic Mexican varieties of *J. curcas* to Indian climatic conditions may result in poor growth performances along with decrease in total seed yield and oil content. However, molecular breeding of non-toxic Mexican population with the three populations of *J. curcas* from North-East India (IITJC7, IITJC12 and IITJC15) having high seed yield traits but comparatively low seed phorbol ester content may result in generation of *J. curcas* varieties having high seed yield traits but negligible seed phorbol ester content. These varieties will be suitable for commercial cultivation under Indian climatic conditions.

The estimation of genetic relationship between *Jatropha* species is imperative for successful inter-specific hybridization of *Jatropha*. The nrDNA ITS and cpDNA *matK* region based assessment of interspecific relationship of *Jatropha* can provide valuable guidelines for further studies related to molecular evolution of *Jatropha* species and their geographic origin. Furthermore, the substitutions and indels obtained in ITS and *matK* region of *J. curcas*, *J. podagrica* and *J. gossypifolia* can be used in future as potential markers for species identification.

Thus it can be concluded that the present study collating morphological traits, molecular markers and biochemical character will provide valuable guidelines for *J. curcas* improvement ventures in India.

Chapter 8

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List of Publications

1. **Basu A**, Mazumdar P, Sahoo L (2011) Genetic diversity among *Jatropha curcas* accessions as revealed by RAPD and Cytochrome P450 gene based markers. National Symposium on “Recent advances in plant tissue culture and biotechnological researches in India” and XXXII Annual meet of plant tissue culture association India, February 4- 6th
2. **Basu A**, Mazumdar P, Sahoo L (2010) Genetic diversity analysis of *Jatropha curcas* germplasm of North-East India. National Symposium on “Plant Cell, Tissue and Organ Culture: The Present Scenario” and XXXI Annual meet of plant tissue culture association India, Kolkata

MANUSCRIPTS UNDER PREPARATION

1. *Jatropha* as a 21st century biodiesel plant: Present status, challenges and future perspectives
2. Morphometric and molecular characterization of *Jatropha curcas* germplasm from North-Eastern India for identification of Candidate Plus Tree
3. Molecular phylogenetics based analysis of *Jatropha* species of North-Eastern India inferred from nuclear ribosomal spacers and chloroplast markers