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Research article

Development of microsatellite, gene-specific and species-specific markers for hybrid detection of plants in *Jatropha* genus

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Abstract

Importance of the work: *Jatropha curcas* has been identified as a potential biodiesel source. It has a narrow genetic base and lacks cultivars with a high seed yield and oil content. Therefore, interspecific hybridization and hybridity testing are important for its genetic improvement.

Objectives: To develop three specific DNA markers and to test their efficiency for hybrid detection of plants in the genus *Jatropha.*

Materials & Methods: Microsatellite, gene-specific and species-specific markers were developed and used to confirm hybrids from interspecific hybridization for the crosses *J. curcas* × *J. integerrima*, *J. curcas* × *J. multifida* and *J. curcas* × *J. podagrica*.

Results: In total, 22 microsatellite primer pairs, 18 single strand conformational polymorphism primer pairs specific to 11 genes and 25 species-specific primer pairs were designed and used to verify interspecific hybrids. All three types of DNA markers developed were effective for the identification of hybrids from interspecific hybridization.

Main finding: The development of the three specific DNA markers for plants in the genus *Jatropha* provided useful information for genetic analysis, hybrid selection and the development of biodiesel plant breeding.

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Introduction

Currently, fossil fuels are being depleted at a fast rate due to the excessive use of energy; furthermore, these fuels are not environmental-friendly (Choo and Ma, 2000). Hence, it is imperative to replace these fuels with environmental-friendly renewable energy resources. More recently, plant-based biofuels have been considered as a replacement for fossil fuel, with the most common biofuels today being ethanol and biodiesel (Choo and Ma, 2000). Biodiesel is an efficient, clean and 100% natural alternative to diesel fuel being better than diesel fuel in terms of sulfur content, flash point, aromatic content and biodegradability (Demirbas, 2003). *Jatropha curcas* or physic nut is gaining importance for biodiesel production because it can be grown in low rainfall areas and in prolonged rainless periods and its oil is regarded as a potential fuel substitute (Openshaw, 2000). It is a plant with many properties and a wide range of uses, including using the oil to produce soup, medicines and pesticides. Even the seed cake produced as a by-product can be used as an excellent organic fertilizer and as a supplement in animal feed (Gomes et al., 2018). However, the major limitation to expanded *J. curcas* use today is its narrow genetic base and low seed yield (Soonthornyatara et al., 2015). Therefore, genetic improvement in *J. curcas* is needed. Interspecific hybridization has been tried between different species of *Jatropha* with limited success. This may have been due to the reproductive barriers between the species that are classified into pre- and post-fertilization. The nature of the barrier will determine the method used to overcome the crossing problem (Tuyl and Jeu, 2005). Understanding of the biological nature of this crossbreeding barrier will pave the way for successful production of new hybrids. However, such studies are still limited with this species. In Thailand, there are only five species out of approximately 175 species of plants that have been reported in the genus *Jatropha*. *J. curcas* and *J. gossypifolia* are used as medicinal plants, while *J. integerrima*, *J. multifida* and *J. podagrica* are attractive ornamentals (Chayamarit, 2001).

Various types of DNA marker have been developed and used successfully in genetic and breeding activities in several crops. Marker technology provides an efficient tool to investigate genetic variation for accelerated breeding, hybrid and parent selections, studying population structure, mapping and tagging of genes or quantitative trait loci linked to major agronomic and economic traits (Kumar-Yadav et al., 2011). Success in marker development in each individual organism depends on its available genetic information. Generally, co-dominant markers are more informative than dominant markers due to the genetic pattern of homozygotes being distinguishable from that of heterozygotes (Novy and Vorsa, 1996). Among various types of co-dominant markers, microsatellite or simple sequence repeat (SSR) markers are widely used (Karaoglu et al., 2005). SSRs are composed of short tandemly repeated motifs of 1–6 bp units and are found in both eukaryotic and prokaryotic genomes and in both coding and noncoding regions (Field and Wills, 1998; Toth et al., 2000). These markers are of choice due to their co-dominant nature, abundance in genomes, hyper-polymorphism, high reproducibility and high rate of transferability across species and genera (Gupta et al., 2003; Varshney et al., 2005). With a large number of expressed sequence tags (ESTs) available in the public domain, EST-derived SSR marker development is an efficient and cost-effective option (Kumar-Yadav et al., 2011). Because EST-SSR markers identify variation in the transcribed regions in genome, the development of gene-based maps can lead to rapid identification of functional candidate genes and high efficiency of marker-assisted selection (Varshney et al., 2005). Approximately 2–5% of the ESTs in several plant species are reported to contain SSRs suitable for the development of SSR markers (Kantety et al., 2002). Generally, EST-derived SSR markers have lower polymorphic levels than genomic sequence-based SSR markers (Kumar-Yadav et al., 2011). However, EST-derived SSR markers possess advantages, such as easy access, presence in gene-rich regions and a high level of transferability to related species, enabling these markers to serve as anchor markers for comparative mapping and evolutionary study (Rungis et al., 2004; Varshney et al., 2005). EST-based markers have been used successfully in several plant species, such as rye (Xu et al., 2012), yellow sarson (Li et al., 2009) and sea buckthorn (Jain et al., 2010)

Gene-specific or functional markers are derived from polymorphic sites within genes that are directly associated with phenotypic variation (Gupta and Rustgi, 2004; Bagge et al., 2007). These markers can be used for validation of cultivar identity, selection of parental materials and the subsequent selection of lines (McCallum et al., 2008). Gene-specific markers based on single nucleotide polymorphisms (SNPs) or small indels (insertion/deletions) provide information of the exact nature of allelic variants and may provide a high density of markers near a locus of interest (Batley et al., 2003). In the same way, species-specific markers are markers that have been developed specific to the species of interest. Where the species of interest is used as the parent of interspecific cross,

primer pairs specific to any regions in the genome of parental species can be used in polymerase chain reaction (PCR) to confirm their interspecific hybrids. PCR-based markers have been used extensively in genetic analysis of several plants and are preferable for a number of reasons: requirement of only small quantities of DNA, no DNA blotting required and easy preparation for automation (Nagaraju et al., 2001). Saptadi et al. (2020) reported the use of SSR, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers to confirm interspecific hybrids of *J. curcas* × *J. integerrima*. However, there are still very few reports pertaining to interspecific hybrid detection in *Jatropha*.

The aim of the present study was to develop three specific DNA markers (microsatellite, gene-specific and species-specific) and to use the developed markers to confirm hybrids from interspecific hybridization among plants in the genus *Jatropha* available in Thailand. Molecular markers offer several advantages over morphological markers because the former provide data that can be analyzed objectively, leading to a new breeding dimension with respect to the time requirement for the development of new improved crop varieties (Kumar et al., 2009a).

Materials and Methods

Plant materials

Samples consisted of *J. curcas* and other plants in the genus *Jatropha*: *J. integerrima*, *J. gossypifolia*, *J. multifida* and *J. podagrica*. Nine F_1 hybrids were selected, consisting of five samples from the cross *J. curcas* × *J. integerrima*, two samples from the cross *J. curcas* × *J. multifida* and two samples from the cross *J. curcas* × *J. podagrica*. Successive backcross (BC) generations were obtained only from the cross between F_1 (*J. curcas* × *J. intergerrima*) × *J. curcas*. In addition, two samples from each of the BC_1, BC_2 and BC_3 generations were selected. The 15 hybrids used as representatives were selected based on their potential high yields and levels of disease resistance. The various *Jatropha* species and the successive hybrids with desirable characteristics are listed in Table 1 and Fig. 1.

Genomic DNA extraction

Total genomic DNA was extracted from young leaves following the cetyl trimethyl ammonium bromide (CTAB) extraction method with some modifications (Doyle and Doyle, 1990). Leaf tissue (0.1 g) was ground in liquid nitrogen and collected in a 1.5 mL microcentrifuge tube. DNA extraction buffer was added, consisting of 3% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 3.5 M NaCl, 2% PVP (pH 8.0) and 0.2% β-mercaptoethanol. The homogenized mixture was incubated at 60°C for 90 min followed by extraction with an equal volume of chloroform: isoamyl alcohol (24:1). Then, the supernatant was transferred to a new tube. Chloroform: isoamyl alcohol (24:1) extraction was done twice and DNA was precipitated using chilled absolute ethanol. The DNA was washed with 70% ethanol for 10 min and then 90% ethanol for 10 min. The pellet was air-dried and dissolved in an appropriate TE buffer. RN*ase*A (50 µL/mL) treatment was used to remove RNA at 37 °C for 2–3 h. The quality and quantity of the DNA were tested based on 0.8% agarose gel electrophoresis.

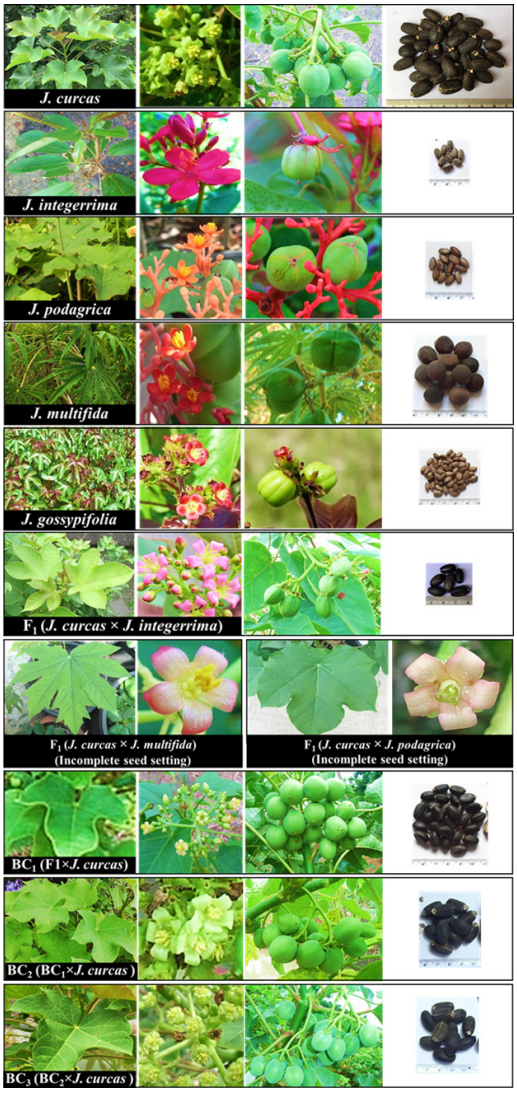

Fig. 1 Samples of plants in genus *Jatropha* and selected hybrids from different generations

Table 1 Morphological characteristics of *Jatropha* species and their hybrids used in this study

Species	Distinct morphological characteristics	Desirable attributes
J. curcas	Tree/shrub, highly branching, cordate-palmately lobed leaves, greenish-yellow flowers, distinct coflorescence, tardily dehiscent fruits with black, large-sized ecarunculate seeds.	Moderate seed yield.
J. gossypifolia	Shrub, profuse branching, cordate leaves, glandular plant parts, dark crimson-purple flowers, violently dehiscent capsules with small brown carunculate seeds.	Drought-tolerance and profuse fruiting.
J. multifida	Shrub/tree, uniform branching, leaves divided into 11 lobes, long petiole, long pedunculated flat-topped cyme, coral-red flowers and non-dehiscent capsules.	Big fruit size and disease-resistance.
J. podagrica	Caudiciform shrubs, cordate leaves with peltate base, flat-topped corymbose cyme, bright scarlet flowers, violently dehiscent capsules with brown ecarunculate seeds.	Fusarial wilt-resistance.
J. integerrima	Shrub, sparse branching, ovate fiddle-shaped leaves, crimson-red flowers, dehiscent capsules, seeds small carunculate and brown with spots.	Semi-hard wood stem and disease-resistance.
F_1 (#1, #4, #5, #12, #137) (*J. curcas* × *J. integerrima*)	Pink or white flowers, complete seed setting and backcrossing to *J. curcas* is possible.	-
F_1 (#189, #192) (*J. curcas* × *J. multifida*)	Pink flowers, incomplete seed setting.	-
F_1 (#242, #253) (*J. curcas* × *J. podagrica*)	Pink flower, incomplete seed setting.	-
BC_1 (#196, #201) (F_1 × *J. curcas*)	Greenish-white flowers, bell-shaped flowers same as *J. curcas*.	Moderate seed yield and moderate disease-resistance.
BC_2 (#403, #423) (BC_1 × *J. curcas*)	Greenish-yellow and bell-shaped flowers, medium height	High number of fruits per plant
BC_3 (#V31, # V36) (BC_2 ×*J. curcas*)	Greenish-yellow and bell-shaped flowers, long peduncle	Large fruit, high number of fruits per plant

= hybrid number from *Jatropha* breeding program based on interspecific hybridization; Information for *Jatropha* species obtained from Basha and Sujatha (2007)

Development of DNA markers for hybridity testing

Microsatellite markers

Primers were designed to flank microsatellite sequences. In total, 72 primer pairs were designed from the *Jatropha* genome database (Hirakawa et al., 2012) and used for assessing hybridity from the crosses *J. curcas* × *J. integerrima*, *J. curcas* × *J. multifida* and *J. curcas* × *J. podagrica*. PCR amplification was done in a thermal cycler (PTC-100TM; MJ Research, Inc.; USA). PCR reaction was performed in a volume of 12.5 µL reaction mixture, containing final concentrations of 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 0.4 µM each primer, 100 ng DNA template and 1 U *Taq* DNA polymerase (Invitrogen; USA). DNA amplifications were carried out with an initial denaturation at 94 °C for 4 min, 35 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 51–65 °C for 1 min and extension at 72 °C for 1.30 min. The final extension was done at 72 °C for 7 min. Equal amounts of formamide dye (98% formamide, 10 mM EDTA, 0.1% bromophenol and 0.1% xylene cyanol) were added to the PCR product and subjected to electrophoretic separation on 6% denaturing polyacrylamide gel in 1× tris-borate-ethylenediaminetetraacetic acid (TBE) buffer. Experiments with each primer pair were replicated three times and those primers producing reproducible fingerprints were reported.

Gene-specific markers

Altogether, 11 specific gene sequences of *Jatropha* were collected from the GenBank database: WRKY transcription factor 58 (WRKY58, KC485310.1), acyl-CoA: diacylglycerol acyltransferase 2 (DGAT2, JQ319813.1), 18S ribosomal RNA - 25S ribosomal RNA (18S-25S, KF500512.1), NAC transcription factor 097 (KC775375.1), carboxyltransferase alpha subunit (AlphaCt, EF095236.1), Enoyl-ACP reductase (FabG, JX111438.1), homomeric acetyl-CoA carboxylase (HCS, JX111449.1), acyl-CoA dehydrogenase (CAD, JX111497.1), Curcin2A (JX111497.1), acetyl-CoA carboxylase BCCP subunit (HQ153098.1) and F-box and leucine-rich

repeat protein (DQ900595.1). All sequences of each gene were compared; gene-specific primer pairs were designed at the conserved regions, mostly in the exons of the gene. In total, 18 primer pairs were designed (Table 5). Then, the primers were tested with 5 *Jatropha* species (*J. curcas*, *J. gossypifolia*, *J. integerrima*, *J. podagrica*, *J. multifida*) and nine samples of F_1 hybrids (Table 1). The reaction was carried out in a volume of 12.5 µL of reaction mixture containing final concentrations of 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 0.4 µM each primer, 100 ng DNA template and 1 U *Taq* DNA polymerase (Invitrogen; USA). Amplification was performed in a thermal cycler (PCR-100TM; MJ Research, Inc.; USA) with a program of initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 53–65 °C for 1 min, extension at 72 °C for 2.30 min and final extension at 72 °C for 4 min. The PCR products were examined using electrophoresis on 1.5% agarose gel known as the DNA fragment length polymorphism (DFLP) technique. Then, the PCR products were examined using the single strand conformational polymorphism (SSCP) technique on 6% non-denaturing polyacrylamide gel (29 acrylamide:1 N, N-methylene bisacrylamide) in 0.5× TBE buffer that had been pre-cooled to 10 °C, at 300 V for about 2–9 h, depending on the length of the PCR product. Before the SSCP analysis, 2–3 µL of SSCP dye (95% formamide, 100 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) were added to the PCR product and then denatured at 95 °C for 2 min, followed by rapid cooling on ice. The gel tank was placed in a tray filled with ice. The gel temperature was kept below 25 °C so that any single-strand secondary structures were maintained during the run. Subsequently, the gel was silver-strained, as described by Caetano-Anollés et al. (1997).

Species-specific markers

Genomic DNA samples of four *Jatropha* species (*J. curcas*, *J. integerrima*, *J. multifida*, *J. podagrica*) were cut using a restriction enzyme (*Mse*I) at 37 °C for 2 h. Then, both ends of the DNA fragments were connected to the *Mse*I adapter (5′-GACGATGAGTCCTGAG-3′/3′-TACTCAGGACTCAT-5′) before using as a template to amplify based on a PCR method using the M1 (M-C) primer (5′-GATGAGTCCTGAGTAAC-3′). Amplification was performed in a 12.5 µL total volume, consisting of 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 1.5 U of *Taq* DNA polymerase (Invitrogen; USA), 1 µM primer and 4 µL DNA template. The reaction was performed in a thermal cycler (PCR-100TM; MJ Research, Inc.; USA), with a temperature profile consisting of an initial denaturation step of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 45 °C, 1 min at 72 °C and a final step of 5 min at 72 °C. The obtained PCR products were connected to the pGEM®-T easy vector (Promega; USA) and white colonies were screened for the inserted DNA fragments based on the colony PCR method. The colonies containing inserted DNA fragments were selected for extraction of their plasmids using a High-Speed Plasmid Mini Kit (Geneaid; Taiwan); the plasmids were sent for sequencing at 1st BASE, Malaysia. Then, species-specific primer pairs were designed from the obtained sequences and used in the PCR amplification of genomic DNA samples of the four *Jatropha* species to confirm the primer specificity before using for hybridity testing. The PCR reaction was performed in a volume of 12.5 µL of reaction mixture containing final concentrations of 0.2 µM of each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 100 ng of genomic DNA and 0.625 U *Taq* DNA polymerase (Invitrogen; USA). Amplification was carried out in a thermal cycler according to a PCR step-cycle program of pre-incubation at 94 °C for 10 min, 34 cycles consisting of denaturation at 94 °C for 1 min, annealing at 51–65 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. To avoid any unequal amplification, due to some of the products being barely visible because of having co-amplified multiplex mixture primers in one reaction, amplification was performed separately using each species-specific primer pair and then the PCR products were combined (for hybrid samples) and examined using electrophoresis on 1.5% agarose gel.

Results and Discussion

Development of microsatellite markers

In total, 72 primer pairs were designed based on general primer design principles using the FastPCR computer program (Kalendar et al., 2017). Microsatellite sequences could be classified into groups of dinucleotide repeat, trinucleotide repeat, tetranucleotide repeat and compound repeat. The dinucleotide repeat was the most common type (58.33%), followed by trinucleotide repeat (23.61%), compound repeat (13.89%) and tetranucleotide repeat (4.17%), respectively. The highest repeat type was dinucleotide repeat $(TA)_n$ (52.78%), followed by trinucleotide repeat $(TAA)_n$ (12.50%) and $(ATT)_n$ (8.33%). The compound repeat $(TG)_n$ $(TA)_n$ was found at approximately 5.56% of all microsatellite sequences (Table 2).

Table 2 Different types of microsatellite base sequences found in microsatellite marker development of *Jatropha curcas*

Microsatellite type	Amount	Percentage
Dinucleotide repeat (58.33%)		
$(TA)_n$	38	52.78
$(CT)_n$	1	1.39
$(GA)_n$	1	1.39
$(TC)_n$	1	1.39
$(CA)_n$	1	1.39
Trinucleotide repeat (23.61%)		
$(TAA)_n$	9	12.50
$(ATT)_n$	6	8.33
$(GAA)_n$	1	1.39
$(CTT)_n$	1	1.39
Tetranucleotide repeat (4.17%)		
$(TTTA)_n$	2	2.78
$(CATT)_n$	1	1.39
Compound repeat (13.89%)		
$(TG)_n (TA)_n$	4	5.56
$(TA)_n (CA)_n$	1	1.39
$(CA)_n (TA)_n$	1	1.39
$(TA)_n (GA)_n$	1	1.39
$(TA)_n (TC)_n$	1	1.39
$(CT)_n (TA)_n$	1	1.39
$(GAT)_n (GAC)_n$	1	1.39
Total	72	100

After testing the primer pairs using the PCR method, 53 primer pairs producing clear DNA bands were selected for DNA fingerprinting of 15 samples from the F_1, BC_1, BC_2 and BC_3 generations (Table 1). The microsatellite marker is the co-dominant marker and allele differences of this marker are based on DNA fragment sizes estimated on denaturing polyacrylamide gel. One DNA band (and stutter bands) can be seen in one allele and in homozygotes, whereas two DNA bands (and stutter bands) can be seen in two alleles and in heterozygotes. The two alleles at a locus in the offspring are inherited, one from each parent, therefore, two different parental DNA bands are observed in the true hybrid. In total, 22 primer pairs could be used to verify hybrids between the three crosses. From the results, 18, 13 and 6 primer pairs were used successfully to identify the hybrids *J. curcas* × *J. integerrima*, *J. curcas* × *J. multifida* and *J. curcas* × *J. podagrica*, respectively. In summary, 5 primer pairs (SSR_JC3, SSR_JC4, SSR_JC9_, SSR_JC38, SSR_JC51) could be used to identify hybrids from all three interspecific crosses in this study. The primer sequences and results of hybridity testing of the three crosses are summarized in Table 3 and Figs. 2A–2B. It is known that replication slippage can occur during *in vitro* amplification of microsatellite sequence and will appear as a minor that differs in size from the main product called 'stutter bands' or 'shadow bands' (Hosseinzadeh-Colagar et al., 2016). An example of stutter bands is shown in Fig. 2B in *J. curcas*, *J. multifida* and their hybrid using the SSR_JC3 marker. However, this marker can still be used effectively for hybridity testing.

Development of gene-specific markers

In total, 18 primer pairs were designed from the *J. curcas* database available in GenBank following general primer design principles. The primers were tested with 5 *Jatropha* species (*J. curcas*, *J. gossypifolia*, *J. integerrima*, *J. podagrica*, *J. multifida*) using a PCR method. In total, 10 primer pairs could be used to amplify the DNA samples of all five *Jatropha* species and eight primer pairs could amplify the DNA samples of some *Jatropha* species (Table 4). The PCR products were tested on agarose gel (DFLP technique) to examine the DNA fragment sizes of the different *Jatropha* species. Where DNA fragment sizes were very similar and could not be distinguished on agarose gel (Fig. 3A), the PCR products were investigated on non-denaturing polyacrylamide gel (SSCP technique) to examine their single strand conformational polymorphism. As offspring inherit two alleles per gene from each parent, different alleles from their parents are important for clear identification of a true hybrid. The allele difference of the SSCP marker is based on not only DNA fragment sizes, but also on the nucleotide sequences in the DNA fragment, which can be examined on non-denaturing polyacrylamide gel, where two DNA bands are evident in one allele and in homozygotes, whereas four DNA bands are evident in two alleles and in heterozygotes.

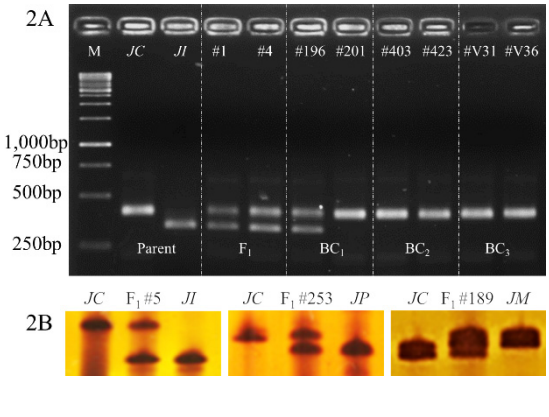

Fig. 2 Examples of DNA banding patterns of parent and hybrids from crosses *Jatropha curcas* (JC) × *J. integerrima* (JI), *J. curcas* (JC) × *J. podagrica* (JP) and *J. curcas* (JC) × *J. multifida* (JM) using microsatellite marker (SSR_JC3, 385bp), where polymerase chain reaction products were examined on 1.5% agarose gel (2A) and on 6% denaturing polyacrylamide gel (2B) and F_1 = first filial generation and BC = backcross generation

Table 3 Developed microsatellite primers for hybridity testing among *Jatropha* species

Primer name	Base sequence (5′-3′)	Length (bp)	Annealing temp. (T_a)	Hybridity testing		
				J. curcas × J. integerrima	*J. curcas* × *J. multifida*	J. curcas × J. podagrica
1. SSR_JC1	F = GAAAGGATATTGTATGACTTTAC R = AATCTCTTGTAGCAGGAATACG	272	59	✓	-	-
2. SSR_JC3	F = GTTGGAGATGATGCATGTGTG R = AACCAATCTATCAAAGAACTAAAC	385	56	✓	✓	✓
3. SSR_JC4	F = AGAAGAGGACCTTACTTTAAAAAG R = AAGTTAAGCATAGTGAATGCAGG	231	53	✓	✓	✓
4. SSR_JC5	F = AAATATGTCATGGTGATGAAGG R = CATGTGATATATTGGCTGAAAG	156	56	✓	-	-
5. SSR_JC9	F = ACTTGTTAGTCCAAAAACTTC R = AAACTCTCCCCTTCTAAAGC	306	56	✓	✓	✓
6. SSR_JC10	F = GATGAGCTATCATGATTATTATTC R = TGTAGAAGAAACCTTATATTGGG	291	51	✓	✓	-
7. SSR_JC11	F = CCCTCTATTTAAAGGCCCTCTG R = ACAGGAGAAAGACCATACCTCC	293	56	-	✓	-
8. SSR_JC13	F = CCACCACCTAAGCTAGCCG R = GTCATCGCCGAGGAAGGTG	229	51	-	✓	-
9. SSR_JC14	F = AGTCTTTTCCCATCTAGTATAG R =AAAGGCAAATAAATGTCACAAC	287	62	✓	-	-
10. SSR_JC23	F = GATATAGGAATCACTTTATATACC R =AAATTGATTTTTGACCTTGCTTC	214	53	-	✓	-
11. SSR_JC26	F = CTCACACATGGCCCATCC R = AATTCGAGGAGATTGGCGCAC	231	65	✓	-	-
12. SSR_JC36	F = AGCTTCTTCTATGCTTTTAGGG R = AAGTACTATTAGTTAGCTTCTAG	200	56	✓	-	-
13. SSR_JC38	F = TGTTTTTAGTGCTGATTTTAATTC R = TGAATGATATACATTGGTTTTGAC	205	53	✓	✓	✓
14. SSR_JC39	F = CAGATTAGGTTGCCCATAGG R = GCAACCCTGCAAAGTTACTG	290	56	✓	-	-
15. SSR_JC40	F = AAACTCTGCCAGTACTGCAC R = TTCCATGAAAAAAGAGAGAACG	243	59	✓	✓	-
16. SSR_JC51	F = AGACGGAGTTAATTTTTTTAGAG R = CTTTACGCAATTGAATGATGTC	201	56	✓	✓	✓
17. SSR_JC63	F = AAAAGTCTCTCACCTTTTGTTG R = CTCAGTTTGGTAGCGGGAC	228	62	✓	✓	-
18. SSR_JC64	F = ACAAATGTTGTATTTGTGGACC R = AGCAGCCAAATGAATTTATAATG	354	62	✓	-	-
19. SSR_JC65	F = CTTTACGGAAATAAACAGAGCC R = AAACACAATAGTAAGTAGAAACG	202	59	-	-	✓
20. SSR_JC67	F = ATGCATAATTTCGCAGATGTTG R = CTTGTCTATGTCAAGAACAAAG	197	59	✓	-	-
21. SSR_JC69	F = TATCTGAGTGATAATTAGAAGAG R = ACACTATGCAGATAATAGTTAAG	245	59	✓	✓	-
22. SSR_JC71	F = GTTTCAACCTGAGCTTAAAGG R = GAAAATTTGTTTCCTTCTTCTAC	182	59	✓	✓	-
Total				18	13	6

✓ = primer pairs that can be used for hybridity testing

Table 4 Developed gene-specific primers and polymerase chain reaction tested results with *Jatropha* species

Gene/Primer name	Base sequence (5′-3′)	Length (bp)	Annealing temp. (T_a)	*Jatropha* species				
				J. curcas	J. gossypifolia	J. integerrima	J. podagrica	J. multifida
1. WRKY58_1	F = ATGAGTGAAGTTTCCTCCCC R = CTCTATTTTCCTGATCTTCAAC	932	53	✓	✓	✓	-	✓
2. WRKY58_2	F = GTTGAAGATCAGGAAAATAGAG R = GCATGCCAAAATTTCTTTCTCC	882	56	✓	✓	✓	✓	✓
3. DGAT2_1	F = ATGGTAGGCGGAGATGGC R = CCCGATCAGAACGAAAGGC	529	56	✓	✓	✓	✓	✓
4. DGAT2_2	F = GTTCTGATCGGGCTTATG R = GAACTCCACCAGGCACCAC	851	59	✓	✓	✓	✓	✓
5. DGAT2_3	F = GTGGTGCCTGGTGGAGTTC R = CCATAGCAGAAAACTGGAACC	751	59	✓	✓	✓	✓	-
6. DGAT2_4	F = GGTTCCAGTTTTCTGCTATGG R = ATGGTAGGCTGTGGATTTTTC	249	56	✓	✓	✓	-	✓
7. (18S-25S)	F = AGGATCATTGTCGAAACCTGC R = CTCCAGACTACAATTCGGAC	311	65	✓	-	-	-	-
8. NAC transcription factor 097 gene_1	F = ATGCGTCCTCCAACAAGTCC R = CACAGGTTTGCTTGTATCATTG	627	56	✓	✓	✓	✓	✓
9. NAC transcription factor 097 gene_2	F = CAATGATACAAGCAAACCTGTG R = TTAGAAGAGGAAGTGAAGTGGC	932	53	✓	✓	✓	-	✓
10. AlphaCt	F = GGTATTCCCCTTGATACGTG R = GACAGTAGGATACGATACATTC	882	56	✓	✓	✓	✓	✓
11. FabG	F = GAGCTTGTCGAATCAGTGAAG R = AACATAACAGAAGTAGTGAGAG	529	56	✓	✓	✓	✓	✓
12. HCS	F = GCTGGTGAGCTTATAGCACG R = TAAGATCTTTGGGAAGGCGAG	594	59	✓	✓	✓	✓	✓
13. CAD	F = GGAGCTGGCAAAGAAAGAAG R = TTCCCATATCGCAGCAATACC	751	59	✓	✓	✓	✓	-
14. curcin2A gene	F = GACCATCTCTCGCTCTCTTC R = CTTTCCACCTTTCATATTGATTTC	249	56	✓	✓	✓	-	✓
15. acetyl-CoA carboxylase BCCP subunit gene_1	F = GTCTGCAGCCTGGTCATAAC R = CACAAGGCTTGCAACCTGGG	311	65	✓	-	-	-	-
16. acetyl-CoA carboxylase BCCP subunit gene_2	F = CCCAGGTTGCAAGCCTTGTG R = ATTCATTAATTTCATGGCTTCAATG	627	56	✓	✓	✓	✓	✓
17. acetyl-CoA carboxylase BCCP subunit gene_3	F = CTGATCAATCAGGAACCATAG R = AAACATGGAAAGTTAAAGTTATTC	400	56	✓	✓	✓	✓	✓
18. F-box and leucine-rich repeat protein gene	F = CATGGTTTTAAAGGCTTGAGG R = GCATTCCACTATCAGTAATAC	429	56	✓	✓	✓	✓	✓
	Total			**18**	**13**	**14**	**13**	14

✓ = primer pairs that can be used to amplify DNAs of *Jatropha* species

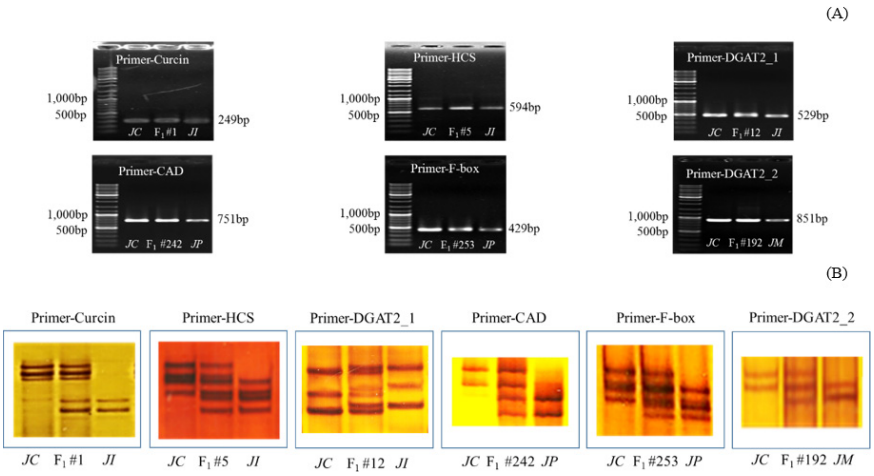

Fig. 3 Examples of DNA banding patterns of parent and F_1 hybrids from crosses *Jatropha curcas* (JC) × *J. integerrima* (JI), *J. curcas* (JC) × *J. podagrica* (JP) and *J. curcas* (JC) × *J. multifida* (JM) using gene-specific markers, where polymerase chain reaction products were examined on: (A) 1.5% agarose gel; (B) 6% non-denaturing polyacrylamide gel

Some gene-specific primers developed in the current study amplified DNA not only in the specific gene, but also in the gene family, resulting in more than two DNA bands being present in both parents and all DNA bands from each parent being present in the hybrid. Furthermore, the sensitivity of the non-denaturing polyacrylamide gel was not high enough to clearly distinguish all single-strand DNA from the gene family, resulting in one DNA band observed on the gel perhaps containing more than one single-strand DNA. An example of DNA banding patterns of hybrids and their parents on agarose gel and non-denaturing polyacrylamide gel using six primer pairs are shown in Figs. 3A and 3B. Considering non-denaturing polyacrylamide gel, the primers curcin 2A, CAD and DGAT2_2 produced two DNA bands (one allele) in both parents and four DNA bands (two alleles) in the hybrid. The primers HCS and F-box produced four DNA bands in both parents and all DNA bands from both parents were found in the hybrid, indicating two independent gene loci were amplified by both primers. Similarly, the primer DGAT2_1 produced three DNA bands in both parents and all DNA bands from both parents were found in the hybrid. Three independent gene loci might be used to explain the obtained DNA banding patterns from the primer DGAT2_1, by considering each DNA band in both parents containing two single-strand DNAs that could not be separated clearly on the non-denaturing polyacrylamide gel. In summary, all 18 primer pairs could be used to amplify different parent-specific bands and to confirm and select true hybrids based on shared bands between parents (*J. curcas* and *Jatropha* species).

Development of species-specific markers

In total, 75 primer pairs were designed from the obtained sequences after sending the plasmids containing the inserted DNA fragments of *J. curcas*, *J. integerrima*, *J. multifida* and *J. podagrica* for sequencing. After testing based on the PCR method, 67 primer pairs provided clear DNA bands and only 25 primer pairs were specific to individual *Jatropha* species. Examples of specific primer pairs are shown in Fig. 4, where DNA bands appear in only one *Jatropha* species. The 25 primer pairs, representing 33.33% of the total designed primers, consisted of 7 primer pairs specific to *J. curcas*, 5 primer pairs specific to *J. integerrima*, 7 primer pairs specific to *J. multifida* and 6 primer pairs specific to *J. podagrica* (Table 5).

Then, the species-specific primers were used to identify the hybrids *J. curcas* × *J. integerrima*, *J. curcas* × *J. multifida* and *J. curcas* × *J. podagrica* using the PCR technique. For hybrid samples, amplification was carried out separately using each primer pair specific to each parent. The combined PCR products from the two amplifications of the hybrid samples were examined on 1.5% agarose gel compared to their parents. Two DNA bands specific to each parent were observed in the true hybrid. It was found that 25 primer pairs could be used to verify the hybrids effectively (Fig. 5).

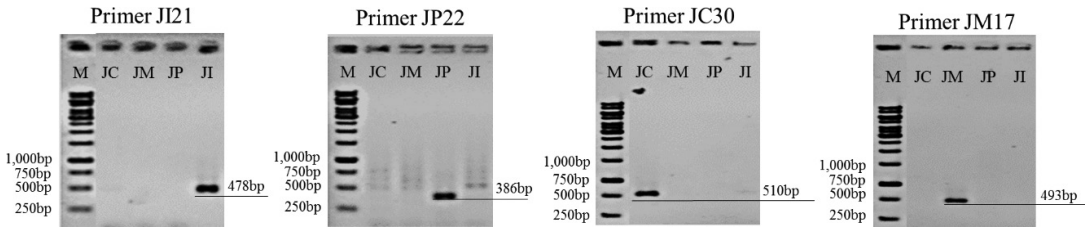

Fig. 4 Agarose gel (1.5%) electrophoresis of plants in genus *Jatropha* using species-specific markers, indicating polymerase chain reaction species-specific amplification where JC = *J. curcas*, JI = *J. integerrima*, JP = *J. podagrica* and JM = *J. multifida*

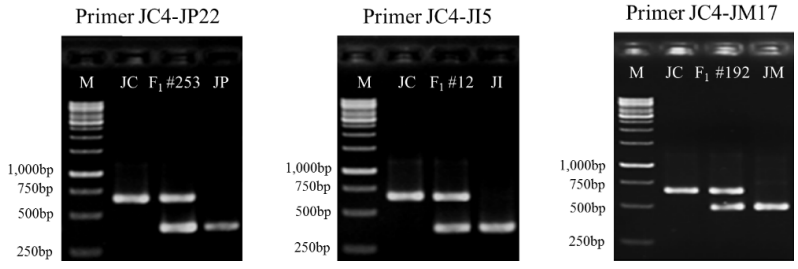

Fig. 5 DNA banding patterns of parent and F_1 hybrids from crosses between plants in genus *Jatropha* using two species-specific markers, where polymerase chain reaction products were combined and examined on 1.5% agarose gel and JC = *J. curcas*, JP = *J. podagrica*, JI = *J. integerrima* and JM = *J. multifida*

Table 5 Developed species-specific primers specific to *Jatropha* species

Primer name	Base sequence (5′-3′)	Length (bp)	Annealing temperature (T_a)	Species specificity
1. JC4	F = CAAAGAATGAATCCCTTTTTTAC	655	59	*J. curcas*
	R = TGATAATAAGAGAAGTACCTGG			
2. JC7	F = AGTGACTATCGATAAGCTGAAG	721	65	
	R = GGGGAATCGACTATCAGTCC			
3. JC27	F = ACATTTGCTTCTCTTACATAGG	673	65	
	R = CATCAGGAGGAAGAGCCAG			
4. JC28	F = AATTTGGTTGGCCTGAAACAG	517	59	
	R = TTCAAATCTGCAATTTCTTTGAG			
5. JC29	F = GTGACTATCGATAAGCTGAAG	720	62	
	R = GGGAATTCGACTATCAGTCC			
6. JC30	F = GGAATTATTTGATTTAGAGCCTG	510	62	
	R = ATTTCAAGACCGGGGTATGTC			
7. JC35	F = TGCCAGTACATTAGCTATTCC	683	62	
	R = ATCTTCACTGTTTGCCTTTTAC			
8. JI3	F = AAAGGACCCAAAAAATTAG	622	53	
	R = TATGGCCTCAGAAACATTCAC			
9. JI5	F = CAAAAAAACTTGAGAACATGTATC	350	59	
	R = CATGTAACTGTATAGCTCCAG			*J. integerrima*
10. JI8	F = TGATTGTGAGAATTCAAAGCC	832	62	
	R = AGACTCTTGGATAGCACTAAC			
11. JI21	F = CTGAGGTTCAGAAGCAGTTG	478	62	
	R = CGTGAAAAGAACTTCTCCAAC			
12. JI22	F = AAGTACTCAACATAGGGTTTC	809	59	
	R = CTTTTTGGCAATCATCATTCCA			
13. JM1	F = AGGAGCAAATCGAAAAGTACC	323	62	*J. multifida*
	R = GAAATCCACGAAAGTCTCAG			
14. JM5	F = GGAACTAATTTTTGTTTTGCATC	360	59	
	R = CATTTTTTTAGCAAGAAGAGATC			
15. JM8	F = GTCATCTCAGGAAGGGACTG	468	62	
	R = AAATAGTATTGGGGCACCCC			
16. JM13	F = TATTTGAGGAATATTTGGAGCC	351	59	
	R = GTTTGACATCCTCCAAGTTAC			
17. JM17	F = CCTTCAATTCTCTAGCCTTC	493	62	
	R = GCCTGTCAAAATACATCTCC			
18. JM19	F = TATTTGAGGAATATTTGGAGCC	351	51	
	R = GTTTGACATCCTCCAAGTTAC			
19. JM20	F = GACATCTAGGGAGATGTTGC	445	59	
	R = CTCCAATACTTATTCATGAACC			
20. JP2	F = CCATGGAGCACCCCAGCC	667	65	*J. podagrica*
	R = TCAAAAACGAGGCAGCTTATG			
21. JP3	F = TACCCCTTATTGTAGGAGCG	619	59	
	R = GAGGAGTGCCAAAGCGTAG			
22. JP9	F = AGTGGGCCTAGCTTTATATC	401	56	
	R = CTGACTCAACCTCCTTATAG			
23. JP17	F = GGGTAAAACTCTACGATAATAG	675	62	
	R = TCTCGTGGTACCCTTCGG			
24. JP19	F = TCAAGCATAAACATAGTGAAAAG	329	59	
	R = CTTCAGGTCTTATTGGCAAC			
25. JP22	F = GTGAATGAAAGATTGATTTTTGG	386	59	
	R = CCTGGAGTTCTTCTAACTCTC			

Microsatellite and gene-specific markers were developed based on sequences of *J. curcas* from the *J. curcas* genome database available in GenBank, whereas species-specific markers were developed based on cloning and sequencing DNA fragments of the individual *Jatropha* species. These three types of DNA markers could be used effectively to verify the hybrids *J. curcas* × *J. integerrima*, *J. curcas* × *J. multifida* and *J. curcas* × *J. podagrica* in the current study. In summary, 5 microsatellite markers (highlighted in the shaded box in Table 3) could be used to amplify all parents in all 3 interspecific crosses, whereas 10 gene-specific markers (highlighted in the shaded box in Table 4) could be used to amplify all 5 *Jatropha* species in this study. These primers should be selected as a priority in further study due to their consistent amplification

across different *Jatropha* species. Polymorphisms based on microsatellite and gene-specific markers among individual *Jatropha* species indicated allele difference at the same locus, while polymorphisms based on species-specific markers among individual *Jatropha* species indicated sequence difference at any regions specific to individual *Jatropha* species. The variations revealed through microsatellite and gene-specific markers can be caused by several events, such as deletion, insertion and base substitution at the same locus. Therefore, these two markers can also be used for genetic diversity analysis of plants in the genus *Jatropha*.

Information on hybridity testing and genetic study of plants among *Jatropha* species using various co-dominant DNA markers is still limited. Sathaiah and Reddy (1985) used isozymes to characterize individual *Jatropha* species. Their study revealed different protein profiles for the four *Jatropha* species used in their study. Additionally, Basha and Sujatha (2007) confirmed the interspecific hybrid of *J. curcas* × *J. integerrima* and evaluated genetic variability between toxic and non-toxic Mexican *J. curcas* using the RAPD technique. The extent of population-level genetic diversity between and within *J. curcas* was also studied using RAPD along with ISSR analysis (Basha and Sujatha, 2007). Pamidimarri et al. (2009) used RAPD and amplified fragment length polymorphism markers to study genetic variability and establish phylogenetic relationships among *J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica* and *J. tanjorensis*. The results showed that the highest genetic correlation was found for *J. curcas* and *J. integerrima*. Attempts have been made to obtain the cross *J. curcas* × *J. integerrima* (Dhillon et al., 2009). The interspecific hybridization was successful, with their hybrids exhibiting a wide range of variation in vegetative traits, such as stem type, branching habit, leaf size and shape, which were similar to those of the parent breeds. Kumar et al. (2009b) assessed the pre- and postzygotic barriers through the pollen-pistil interaction of *J. curcas* and *J. podagrica*. The results exhibited normal pollen tube growth; however, the growth rate was slow and reached the ovary 4 h after pollination. These crosses produced seeds, but the seed set percentages were very low and no seed germinated at all. Similar results were obtained from the current study on interspecific hybridization that produced F_1 hybrids from the crosses *J. curcas* × *J. integerrima*, *J. curcas* × *J. multifida* and *J. curcas* × *J. podagrica*. While many backcross progenies were derived from the F_1 hybrids of the cross *J. curcas* × *J. integerrima*, no backcross progeny were derived from any of the other interspecific crosses.

Conclusion

Co-dominant markers, which generally are more informative than dominant markers, were developed and could be used effectively to confirm hybrids from interspecific hybridization. The development of the three specific DNA markers for plants in the genus *Jatropha* should be useful for genetic analysis, hybrid detection and further success in *J. curcas* improvement project.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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