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Development of Interspecific Hybrid of *J. curcas x J. integerrima* and its Molecular Characterization using SSR Markers

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Abstract—*The successful crosses were attempted between J. curcas L. (2n=22) and J. integerrima (2n=22). The interspecific hybrid exhibited wide range of variation on morphological and shows intermediary of parents in term of fruits and leaves. A total of 15 SSR markers were used for the investigation of true hybrid with their parents (J. curcas and J. integerrima). Jaccard's pairwise similarity coefficient values ranged from 0.06 to 0.99. The dendrogram were grouped in two major clusters. The maximum hybrids grouped in the cluster-I with J. curcas that showed the more similarity with female parents. The minimum genetic similarity coefficient was found between J. curcas and J. integerrima (0.06%). In cluster-II, male parent, J. integerrima, was out- grouped from the Hybrids-NBJIS- 1, 2, 15, 48 and 14. Characterization of advanced generation interspecific derivatives of J. curcas and J. integerrima cross carried out in the present investigation indicate ample scope for genetic enhancement of J. curcas through interspecific gene transfer.*

Keywords*: Jatropha, Interspecific hybridization, Hybridity assessment, SSR markers.*

1. INTRODUCTION

J. curcas L. is belonging to the family Euphorbiaceae having chromosome number 2n=22 [4] with relatively smaller genome size of ~416 Mb [3, 19]. *J. curcas* L. is a tropical species native to Mexico and Central *America*, but widely distributed in other tropical and sub-tropical areas of the world, especially in Africa, India and South-East Asia ([8, 20, 13]. *J. curcas* has received much attention as promising oilseed crop for biofuels production among not a food crop [8, 13, 22], except for some low-phorbol ester varieties from Mexico that can be cooked as snacks [24]. Despite the potential of *Jatropha*, the crop productivity is far too low due to limited genetic diversity, low seed yield, susceptible to many insects, pests and viral diseases effects of environment and genotype x environment interaction [7, 18, 14]. Genetic improvement of *J. curcas* can be practiced through many options like traditional breeding, heterosis breeding [23], mutation breeding, interspecific hybridization and genetic transformation [6].Some preliminary interspecific hybridization program has been conducted by some researchers among different species of *Jatropha* with limited success [4, 20]. Successfully development of interspecific hybrid and crosses compatibility was observed by different researchers proved that the crosses of J. curcas and *J. integerrima* is more successful than other crosses combinations [21,5]. The interspecific hybrid play key role for the improvement *J. curcas* with low phorbolester (PE) [16]. The interspecific hybrid of *J. curcas* from Mexico and *J.integerrima* form Thailand as a new promising source of woody biomass [12]. EST-SSR markers were efficiently used for the identification of interspecific and intergeneric hybrids among Jatropha-related species [9]. Recently, some interspecific *Jatropha* was developed with enhanced growth, yield and oil attributes suitable for semi-arid wastelands [1]. However, the interspecific hybridization program and hybridity assessment in *J. curcas* is very limited. Therefore, our present investigation interspecific hybrids were developed between *J.curcas* and *J.integerrima*. The hybrids were verified using SSR markers developed earlier [11].

2. MATERIALS AND METHODS

2.1 Plant materials Genomic DNA isolation

Interspecific hybridization was made between a large canopy type of of *J. curcas* (Jc) as female and an erect and tall canopy type of *J. integerrima* (Ji) as male from India. In the direct cross, 300 female flower of *J.* curcas were emasculated before anthesis at evening (4:00 PM to 6:00 PM) and hand-pollinated by pollen of *J. integerrima* during 8:00-10:00 PM. The interspecific hybridization experiment was conducted in the year October 2011 to January, 2012 in a field of CSIR-NBRI, Lucknow. The resulting hybrid seeds were germinated in plastic bags and maintained at CSIR-NBRI experimental glass

house in April, 2012. The mature 120 hybrid seedlings were transferred into field in July, 2012 and only 94 hybrids successfully survive. The hybridity was confirmed early seedling stage using SSR markers developed by [11]. The genomic DNA was extracted from young leaves of parent (*J. curcas* and *J. integerrima*) and 94 interspecific hybrids by CTAB (Cetyl Trimethyl Ammonium Bromide) method. The quality of DNA was checked on 0.8% agarose gel, and DNA concentration was determined using a Nanodrop spectrophotometer ND1000 (Nanodrop Technologies, DE, USA).

2.2 Morphological evaluation of F_1 hybrids

The hybrid nature of F_1 plants were evaluated by visual morphological observation of parents and their hybrid plants on the basis of leaf shape and size, leaf pigmentation, fruit size, stem type and flower colour, number of branches, number of inflorescence, number of male and female flower/inflorescence.

2.3 Microsatellite analysis

PCR amplification was carried out in 10µl reaction mixtures that contained 10 ng genomic DNA, 1X PCR master mix (AmpliTaq Gold®, Applied Biosystems, USA), 0.1 µl (5pmol/µl) of forward primer (tailed with M13 tag), 0.3µl (5pmol/µl) each of both reverse primer and M13 tag (labeled with either 6- FAM, VIC, NED and PET). PCR was performed on Verti Thermal Cycler (Applied Biosystems, USA) using the following cycling condition: initial denaturation at 95^0C for 5 min followed by 36 cycle of 94^0C for 30 s, 50-55^0C (primer specific) for 45 s and 72 ^{0}C for 1 min. Subsequently, 10 cycles of denaturation for 30 s at 94˚C, annealing for 45 s at 53^0C, extension for 45 s at 72^0C followed by final extension for 15 min at 72^0C was performed. The amplified PCR products from the parents and hybrids were resolved by TBE agarose gel electrophoresis using 1.5% Agarose (Geni) and then post PCR multiplex sets was prepared based on fluorescence labeled primers. For post PCR multiplex set, 1 µl FAM and 2 µl of each VIC, NED, and PET labeled PCR product were combined with 13 µl of water. 1 µl of this mixture was then added to 10 µl Hi-Di formamide containing 0.25 µl GeneScan ™ 600 LIZ® as internal size standard. This was then denatured for 5 min at 95^0C, quick chilled on ice for 10 min and run on a capillary-based 3730xl DNA analyzer (ABI, USA). Microsatellite loci repeats were assayed on the basis of their observed heterozygosity and number of alleles detected with PCR amplification profile. Fragment analysis was performed using GeneMapper software ver.4.0 and data were scored as allele size (bp).

2.4. Statistical analyses

The allelic data of 15 polymorphic SSRs were subjected to statistical analysis using PowerMarker ver. 4.0 [10] to calculate the total number of alleles, allele frequency, major allele frequency (*MAF*), gene diversity (*GD*), expected heterozygosity (*He*) and polymorphic information content (PIC) value. The PIC value was calculated following [2] as follows:

$$PIC = 1 - \left[\sum_{i=1}^{n} Pi^2\right] - \left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2Pi^2Pj^2\right]$$

Where *Pi* and *Pj* are the frequencies of the i^{th} and j^{th} allele.

The 0-1 data matrix was further used to calculate pair-wise genetic similarities among all the accessions using Jaccard's coefficient through NTSYS-PC software v 2.02 [17]. The similarity matrix was then used to generate dendrogram depicting clustering pattern of cultivars using unweighted pair-group method of arithmetic average (UPGMA) methods.

3. RESULTS AND DISCUSSIONS

3.1 Interspecific hybridization

A successful interspecific cross was made between *J. curcas* and *J. integerrima.* (Fig.1). More than 300 crosses were attempted, of which 150 fruits were successfully developed. After maturity 220 hybrid seeds were harvested and shown in polybags in glass house. Of which, 160 seeds were germinated (72.7%) and only 120 seedlings were successfully survive in glass house (Table 1). The two month old seedlings were transferred in the field and 94 seedlings successfully survived at maturity in the field. Previously, successful interspecific hybrid has been developed by the crosses of *J. curcas* and *J. integerrima* [21, 15]. The reciprocal crosses of *J. curcas* and *J. integerrima* was less successful [5].

Fig. 1: Photograph showing (A) *J. curcas* (Female parent) (B) *J. integerrima* (Male parent) selected for interspecific hybridization (C) Developing successful fruits after crossing (D) Seedling of F_1 interspecific hybrids in glasshouse, (E) Close up view of inflorescence of hybrid plant and (F) Hybrid plant showing fruits

Table 1: Crossability success in crosses between *J. curcas* and *J. integerrima*

No. of crosses	No. of capsules formed approx.	No. of seeds set	No. of seeds germinated	Seedlings established in field
300	150	220	160	94

3.2 Morphological characterization of F_1 hybrids

The successfully survive interspecific hybrids were used for the morphological characterization which have been already described by other researchers [21, 5]. The developed interspecific hybrids were found great variations at morphological level such as high vigorousity , freely flowering and morphologically intermediate between the parents in term of leaf pigmentation, fruit size, stem type and flower colour (Fig.1 and 2). In the early stage, a large number of hybrid plant leaves showed dark red pigmentation on ventral surface and at maturity maximum plants leaves became green while some plants leaves retained their colour. Some hybrids showed complete dark red pigmentation on ventral surface, some showed pigmentation of half part and some showed scattered pattern of pigmentation (Fig.2). The hybrid plants flowered within 7-8 months unlike the parents which bore flowers 10-15 months after establishment. The capsule variability were of intermediate type, either round like *J. curcas* but purple colored or oval like *J. integerrima* without pigmentation.

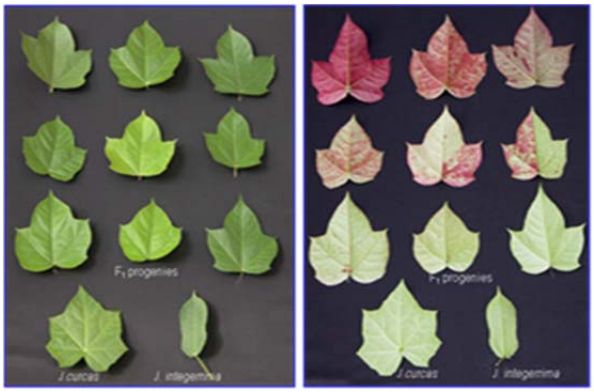

Fig. 2: Morphological variation in leaf shape and pigmentation of parents (*J. curcas* and J. *integerrima*) and hybrid plants

3.3 Hybridity assessment

A total of 94 interspecific hybrids were identified along with their parents at early stage using 15 polymorphic SSRs markers.. A snap shot of GeneMapper V4.0 represented in fig.3 showed the identification of true hybrids. The SSR based allelic variation of all the 94 F_1 hybrids showed in table 2. The maximum similarity of hybrid was found with female parental type (516), while 149 alleles showed similarity with male parental type. A total of 483 alleles were found in interspecific hybrids with sharing both the parental types alleles. The number of alleles (256) showed non-parental type of alleles with hybrid genotypes. Jaccard's pairwise similarity coefficient values ranged from 0.06 to 0.99. The UPGMA based dendrogram were grouped in two major clusters (Fig. 4). The maximum hybrids were grouped with *J. curcas* in the cluster -I that showed more similarity with female parents. The minimum genetic similarity coefficient was found between *J. curcas* and *J. integerrima* (0.06%). In cluster-II, male parent (*J. integerrima*) was out- grouped with the hybrids-NBJIS1, NBJIS2, NBJIS15, NBJIS48 and NBJIS14.

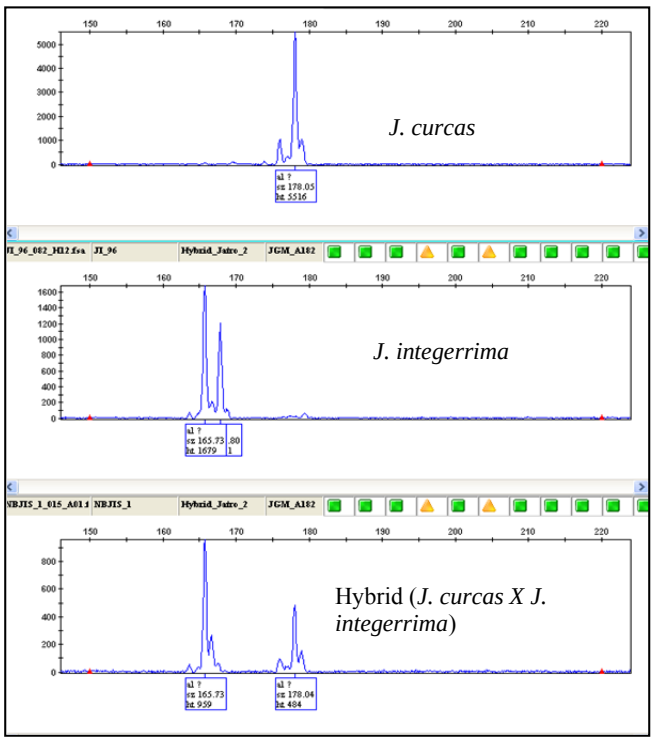

Fig. 3: A snapshot of Gene Mapper showing the allelic pattern of *J. curcas, J. integerrimma* and their hybrid

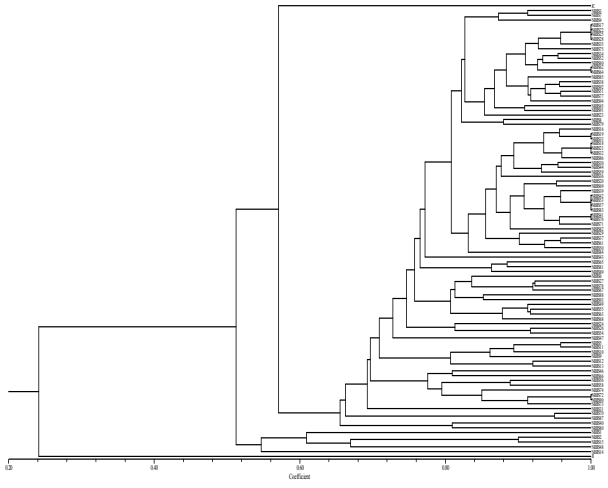

Fig. 4: Dendrogram generated by UPGMA of 94 hybrids with their parents *J.curcsa* and *J.integerrima*.

Table 2: Genotyping details of 15 polymorphic primers with 94 interspecific hybrids and its parents (*J. curcas* and *J. integerrima*)

SSR Name	Allele size J. curcas	Allele size J. integerrima	Number of hybrids with different allele combinations			
			Hybrids both parental type	Hybrids female parental type	Hybrids male parental type	Non-parental type
JGM_A109	244	263	75	9	6	4
JGM_A120	252	245	10	36	6	42
JGM_A162	275	285	56	22	13	3
JGM_A179	175	263	4	81	3	6
JGM_A182	178	166	89	2	1	2
JGM_A247	288	301	80	6	3	5
JGM_A291	290/303	298/303	1	14	55	18
JGM_A295	239	212	13	20	10	51
JGM_A296	192	184	15	31	11	37
JGM_B244	185	183	20	48	6	20
JGM_B278	155	142	25	3	28	38
JGM_B334	167	142	93	1	-	-
JGM_B369	147	142/147	1	88	4	1
JGM_B576	251	243/268	-	93	1	-
JGM_A335	320	291	1	62	2	29
		Total-	483	516	149	256

4. CONCLUSIONS

In conclusion, we have successfully developed interspecific hybrid between *J. curcas* and *J. integerrima* showing good resistant against *Jatropha* mosaic virus and frost. The introgression of desirable traits with number of morphological variations could be used in future breeding program of *J. curcas* for the development of high oil yielding varieties which can play significant role to alleviate the energy crisis.

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