Estimation of outcrossing rates in interspecific backcross plants of *Jatropha curcas* (L.) using AFLP and SSR markers

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Abstract In this paper, we report the estimates of outcrossing rates using open-pollinated progeny arrays of 40 BC₁ individuals of *Jatropha* developed as a result of interspecific hybridization between *J. curcas* and *J. integrerrima*. For analysis PCR-based dominant AFLP and codominant SSR markers were used. The multilocus outcrossing rate (t_m) estimated from AFLP markers (0.892 ± 0.112) are almost in the same range with SSR (0.884 ± 0.293) markers which indicate a high level of heterozygosity. A low value of inbreeding coefficient (F) also points out to the fact that outcrossing was the prevalent mode of reproduction in *Jatropha* and suggests maintenance of adequate genetic variability within families.

Keywords AFLP · Backcross · Biodiesel · *Jatropha* · Outcrossing · SSR

Introduction

Jatropha curcas (family- Euphorbiaceae) has received great attention as a potential source of bio-diesel during the last decade. Several initiatives are in progress to develop improved varieties of *Jatropha* through breeding and selection.

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These breeding programs have been hindered due to a narrow genetic base in this species, especially among accessions from India, Southeast Asia and Africa (Basha et al. 2009; Pamidimarri and Reddy 2014).

The genetic structure of plant populations is determined by the prevalent mating system together with the mechanisms of pollen and seed dispersal (Charlesworth and Wright 2001). Few studies have been reported so far on mating system and outcrossing rates in *Jatropha*. *Jatropha* bears unisexual flowers in dichasial cymes where both male and female flowers are present in the same inflorescence (Raju and Ezradanam 2002). In addition, both, protandry and protogyny have been reported in *Jatropha* (Raju and Ezradanam 2002). Pollination is predominantly mediated by insects such as *Apis dorsata* and *A. mellifera*. So *apriory*, the floral system of *J. curcas* seems to favour outcrossing (Kaur et al. 2011; Wang and Ding 2012). Recently, a mixed-mating reproductive system with up to 68.3 % outcrossing and 13 % apomixis has been reported in *Jatropha* (Bressan et al. 2013).

Outcrossing rates have been traditionally studied using codominant markers such as isozymes and microsatellites due to their high allelic polymorphism (Adugna et al. 2013; Chaix et al. 2003). However, dominant markers such as RAPD and AFLP have also been used successfully especially when microsatellite markers were unavailable (Gaiotto et al. 1997). Due to low genetic diversity in *Jatropha*, detection of outcrossing events may be difficult which may in turn lead to underestimation of outcrossing rates. In this study, we studied the outcrossing rates in an inherently diverse set of individuals using AFLP and microsatellite loci. We also compared the efficacy of the two marker systems for the estimation of outcrossing rates.

Table 1 List of backcross plants used for estimation of outcrossing rates in *Jatropha*

S. No.	Mother plants (BC ₁)	No. of progeny
1	BC ₁ -10	10
2	BC ₁ -11	10
3	BC ₁ -21	10
4	BC ₁ -28	10
Total	4	40

Materials and methods

Plant material

The research material used in this study included 40 open pollinated progenies derived from 4 mother plants (Table 1). The mother plants belonged to a BC₁ mapping population obtained through interspecific hybridization

between *J. curcas* and *J. integerrima* (Sinha et al. 2015a). The BC₁ plants had been planted in a block (2 m × 3 m spacing) which was surrounded by a large number of *J. curcas* accessions. All individual plants of BC₁ block and surrounding blocks were in bearing stage at the time of collection of seeds.

AFLP and microsatellite genotyping

DNA was isolated from young leaves following a modified CTAB based procedure (Singh et al. 1999). AFLP genotyping was done using the protocol described by Vos et al. (1995). The EcoRI primers were end labeled using [γ -³²P] ATP. A total of 4 AFLP primer combinations were used (Table S1). The AFLP fragments were resolved on 6 % polyacrylamide gels and detected by autoradiography. Microsatellite genotyping was done with 8 microsatellite markers (Table S2) as per Sinha et al. (2015b).

Fig. 1 AFLP profile showing outcrossing rates in 4 mother plants with their respective progenies using primer combination E-AGxM-CTA

Data analysis

AFLP bands were scored manually for their presence or absence. The amplicons were in the size range of 50–500 bp. SSR bands were scored for different allelic states in codominant fashion. The program MLTR (version 3.4) was used to calculate the multilocus outcrossing rate (t_m), the single locus outcrossing rate (t_s), the apparent level of selfing due to biparental inbreeding ($t_m - t_s$), the correlation of selfing among maternal plants (r_s) and the multilocus correlated paternity (r_p) using mixed-mating model with 1000 bootstraps (Ritland 2002). The inbreeding coefficient at equilibrium (F_{eq}) was calculated as $F_{eq} = \frac{1-t_m}{1+t_m}$, where t_m is the multilocus outcrossing rate (Fyfe and Bailey 1951).

Results

With 109 polymorphic AFLP markers, none of the progenies were found to be identical to their seed parents (Fig. 1). On the other hand, 52 % of the progenies were identical to their seed parents for all the eight SSR loci (Fig. 2). This indicated that the AFLP markers had higher discriminating power than the SSR markers. The multilocus outcrossing rate (t_m) estimated using AFLP markers was 0.892 ± 0.112 whereas that estimated from SSR data (8 SSRs) was 0.884 ± 0.293 . The mean single

locus outcrossing rates (t_s) were significantly different than t_m in case of AFLP markers but not in case of SSR markers.

The estimated fixation index (F) was -0.023 and -0.2 for AFLP and SSR markers, respectively. On the other hand, the expected F values were 0.057 and 0.061 for AFLP and SSR respectively.

The multilocus correlation of paternity with AFLP markers was 0.142 which indicated involvement of several pollen parents. The correlation of selfing among loci was 0.999 and 0.285 with AFLP and SSR markers respectively (Table 2).

Discussion

Occurrence of apomixis was ruled out in the present study as none of the progenies were genetically identical to their respective seed parent. However, with SSR markers, an average of 52 % progeny individuals had multilocus genotypes identical to their respective parents. In the absence of any additional data, this could have been attributed to apomixis, at least in part. Thus, the AFLP markers displayed greater efficacy than SSR markers in the current study. The difference in inferences from AFLP and SSR data is mainly due to the remarkable difference in the number of informative markers in the two datasets. It may be noted that in the current case, the SSR markers had limited advantage over AFLP markers per locus basis as only two alleles for each SSR (one from

Fig. 2 SSR profile showing outcrossing rates in 4 mother plants with their respective progenies using microsatellite primer JcSSR 2742

Table 2 Mixed mating parameters obtained from MLTR software version 3.4 using AFLP markers, SD = standard deviation, r_p = correlation of paternity

Parameters	AFLP	SSR	AFLP + SSR
Multilocus t estimate (t_m) with SD	0.892 (0.112)	0.884 (0.293)	0.977 (0.113)
Singlelocus t estimate (t_s) with SD	0.784 (0.122)	0.881 (0.292)	0.809 (0.109)
Biparental inbreeding ($t_m - t_s$) with SD	0.108 (0.067)	0.003 (0.033)	0.168 (0.097)
Correlation of t (or s) estimate	0.056 (0.485)	0.999 (0.847)	−0.999 (0.102)
Multilocus correlation of p estimate $r_p(m)$	0.142 (0.387)	0.016 (0.325)	0.188 (0.387)
Singlelocus correlation of p estimate $r_p(s)$	0.143 (0.115)	0.021 (0.329)	0.335 (0.280)
Difference [$r_p(s) - r_p(m)$] of p estimate	0.001 (0.356)	0.005 (0.112)	0.147 (0.345)
Correlation of s (or t) among loci	0.999 (0.635)	0.285 (0.737)	0.999 (0.696)
$F_{estimated}$	−0.023 (0.136)	−0.200 (0.171)	−0.030 (0.147)
$F_{expected}$	0.057	0.061	0.011

J. curcas and the other from *J. integerrima*) were expected in the plants of the entire experimental plot.

Apomictic fruit set and seed production has been reported in *J. curcas* in artificial pollination experiments under field conditions by protecting female flowers. Apomixis rates of 32 to 36 % have been reported from field grown *Jatropha* in India (Bhattacharya et al. 2005) and China (Chang-Wei et al. 2007). However, a much lower level of apomixis (13 %) was inferred from frequency of progeny with identical multilocus genotype to the mother tree (Bressan et al. 2013).

In the current study, *Jatropha* displayed a mixed mating system (AFLP, $t_m = 0.892$; SSR, $t_m = 0.884$), producing both outcrossed and selfed seeds (Table 2). Indeed, the t_m obtained using combined data (AFLP and SSR) was 0.977. The results are in correspondence with the general expectations considering the floral biology and previous reports in *Jatropha* (Bhattacharya et al. 2005; Raju and Ezradanam 2002). A mixed mating system has been observed in *Jatropha* (Bressan et al. 2013). In this study, the multilocus outcrossing rates (t_m) ranged from 0.21 to 1.0 with a mean t_m of 0.683. On the other hand, the level of biparental inbreeding, which is indicative of mating among related individuals, in the current study was lesser (0.168) than the previous study (0.347). These differences in multilocus outcrossing rate and the level of biparental inbreeding in two studies may be attributed to greater efficiency of detecting outcrossing events in the current study due to inherent diverse nature of the research material. A low correlation of paternity may be due to high level of diversity among the BC_1 individuals. A low value of inbreeding coefficient (F) also points out to the fact the outcrossing was the prevalent mode of reproduction in these plants.

An important feature of the current dataset is that the mother plants were highly suitable for study of outcrossing rates. It was expected that a significant marker loci in the mother plants will be in heterozygous state as they belonged to first backcross generation. Therefore, discrimination between selfing and apomixis as well as identification of outcrossing events would be easier. As

almost all the allelic polymorphism in these samples was due to their interspecific origin, no ambiguity in scoring was encountered.

In general, populations with high outcrossing rates exhibit higher genetic variability in comparison to inbreeding populations (Charlesworth and Wright 2001). This condition is inconsistent in case of *Jatropha* where genetic variability is low despite high outcrossing rates. This may be due to founder effect which occurs due to introduction of a population of highly related individuals (Sinha et al. 2013; Wright 1931).

Availability of complete genome sequence of *Jatropha* has now made it possible to use next generation sequencing and other high throughput genotyping methods for genetic studies (Wu et al. 2015). However, these tools are still not cost-effective at per sample basis. On the other hand, per sample costs are much lower with AFLP and microsatellites which can generate adequate number of markers within short time.

The high outcrossing rates observed in *Jatropha* in this study as well as by others shows that seed orchards containing diverse accessions of *Jatropha* can be used for large scale seed production without any significant risk of selfing and consequent inbreeding depression. This would reduce the cost of planting material as compared to vegetative propagation. Use of seed raised plants would also increase the rate of stand establishment and reduce the mortality from color rot and water logging.

Conclusions

This is the first study employing AFLP markers as well as backcross populations for estimation of outcrossing rates in *Jatropha*. Our data indicates that *Jatropha* is a predominately outcrossed species (97.7 %) with very low level of true selfing. Further, no evidence of occurrence of apomixis could be obtained unlike the previous studies.

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