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Full Length Research Paper

Estimates of outcrossing rates in *Moringa oleifera* using Amplified fragment length polymorphism (AFLP)

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The mating system in plant populations is influenced by genetic and environmental factors. Proper estimates of the outcrosing rates are often required for planning breeding programmes, conservation and management of tropical trees. However, although *Moringa oleifera* is adapted to a mixed mating system, the proportion of selfing has not been previously estimated. The current work therefore, shows the use of AFLP markers in a mating system study of *M. oleifera* seed orchard. Data revealed a mixed mating system with a multilocus outcrossing rate (t_m) of 0.74. It further demonstrated that AFLP markers, though dominant with a lower information content than co-dominant markers are adequate for the study of the mating system in plant populations. The 26% selfing observed in *M. oleifera* can lead to overestimation of the proportion of additive genetic variance and appropriate adjustments are therefore required. However, the presence of selfing as well as early sexual maturity (6 months to 1 year) in *M. oleifera* provides an opportunity for developing inbred lines and hybridisation. Additionally, in designing *M. oleifera* seed orchards, randomisation and minimum distance between related individuals need to be worked out to maximise cross-fertilisation among unrelated clones and minimise selfing or mating among related ramets.

Key words: *Moringa oleifera*, mating system, outcrossing rates, amplified fragment length polymorphism.

INTRODUCTION

Moringa oleifera Lam belongs to a monogeneric family of shrubs and trees, the moringaceae (Ramachandran et al., 1980). *M. oleifera* seeds contains flocculants for water purification (Jahn, 1984; Gassenchmidt et al., 1995; Muyibi and Evanson, 1995; Ndabigengesere et al., 1995), antimicrobial substances (Jahn, 1984) and edible oil (Khan et al., 1975; Ramachandran et al., 1980). Other uses of this species are for vegetables, fodder, medicines, gum, food spices, rayon and paper pulp (Jahn et al., 1986; Nautiyal and Venkataraman, 1987; Babu and Rajasekaran, 1991; Jahn, 1991; Mayer and Stelz, 1993; Caceres et al., 1991). Wide scale planting of *M. oleifera* in East Africa has gained momentum over the last four years. Seeds for planting in Kenya, Tanzania and lately Uganda, are being obtained mainly from Mbololo, Kenya. These seed sources have been established and maintained by farmers and information is scarce on their genetic quality.

The mating system in plant populations is influenced by genetic and environmental factors (Clegg, 1980). Proper estimates of the outcrossing rates are often needed for planning breeding programmes (Ritland and Jain, 1981), conservation and management of tropical trees (Loveless, 1992). The majority of outcrossing angiosperms have bisexual flowers, a condition from which self-pollination can evolve directly through the

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modification of self-incompatibility or other floral traits that prevent self-pollination (Schoen et al., 1997). In addition to the role of the variable floral architectures in determining mating systems of the plant populations (Ennos, 1981), the mating system may be sensitive to plant density and population size (Clegg, 1980; Ennos and Clegg, 1982; Goodell et al., 1997), type of pollination vector and abundance (Aide, 1986), flower colour (Brown and Clegg, 1984), size of floral display (Dudash and Barret, 1989) and anther-stigma separation (Karron et al., 1997). Temporal changes in quality or quantity of pollinator service or variation in the timing of flowering can lead to seasonal changes in the mating patterns and composition of the outcross pollen pool (Moran and Brown, 1980; Fripp et al., 1987; Goodell et al., 1997; Mitchell and Marshall, 1998). As such it is reasonable to expect that outcrossing rates could vary extensively both spatially within and between populations, and temporally within a single population (Wolfe and Shore, 1992).

Traditional methods used for the measurement of mating systems have been based on the analysis of floral morphology, greenhouse crossing experiments, and (where appropriate) the observation of pollinator behaviour (Clegg, 1980). The practical use of phenotypic markers in trees is limited by a number of factors such as long time required for progeny to reach maturity for the markers to be scored and lack of consistency between phenotypic markers and outcrossing (Gjuric and Smith, 1996). The development and application of isozymes provided numerous genetic markers which can be used to measure mating systems in plant populations (Brown and Allard, 1970; Holtsford and Ellstrand, 1990; Cottrell and White, 1995; Premoli, 1996; Schoen et al., 1997). In recent years DNA based methods such as RAPDs (Gjuric and Smith, 1996) and AFLPs (Gaiotto et al., 1997) have been used to estimate outcrossing rates. However, due to their dominance behaviour, RAPD and AFLP markers provide less information per locus than co-dominant markers (Gaiotto et al., 1997). This is particularly relevant for applications that require genotype discrimination, as in the case of outcrossing-rate estimation (Gaiotto et al., 1997). However, Ritland and Jain (1981) demonstrated that this limitation could be readily overcome by multilocus estimation of outcrossing with dominant markers having intermediate gene frequencies.

M. oleifera is adapted to selfing (geitonogamy) and outcrossing (xenogamy) with larger fruit set, seed set and fecundity in the latter mode (Jyoth et al., 1990). The flowers produce both pollen and nectar with bees as the main pollinators (Puri, 1941, Jyoth et al., 1990, Chand et al., 1994). However, the proportion of selfing in *M. oleifera* has not been previously estimated. The main aims of this study were to test the utility of dominant AFLP markers in estimating outcrossing rates in *M. oleifera* and then use them (AFLP markers) to obtain estimates of outcrossing rates in an *M. oleifera* seed orchard from Mbololo, Kenya.

MATERIALS AND METHODS

Plant material and DNA isolation

Single tree collection was carried out in an *M. oleifera* seed orchard in Mbololo, Kenya. The seeds were grown under greenhouse conditions. A random sample of 4 families of open pollinated progeny arrays of 20-23 individuals, giving a total of 86 individuals, were used for this study. DNA was isolated following a modification of Edwards et al. (1991) method exactly as used by Muluvi et al. (1999).

AFLP procedure

The AFLP technology was carried out as described by (Vos et al., 1995), employing *Pst*I and *Mse*I as rare and frequent cutter enzymes, respectively. The nucleotide sequence of the two AFLP primer pairs used, *Pst*I (P12, P11) and *Mse*I (M51) together with the corresponding adapters are shown in Table.1. The AFLP markers were identified by the first primer code followed by the locus number, e.g., P12-1.

Data analysis

Scoring of bands was carried out considering only two possible alleles: band presence or absence. The mating system was analysed using the multilocus mixed mating program (MLDT) of Ritland (1990). From progeny array data, the programme simultaneously estimated (i) the multilocus outcrossing rates (t_m) by the Newton-Raphson method; (ii) the mean single-locus outcrossing rate (t_s); (iii) single locus inbreeding coefficient (Wright's fixation index) of the maternal parents (*F*); (iv) the pollen and ovule allele frequencies by the expectation-maximisation method and; (v) variances of the above quantities using the bootstrap method where the progeny array (within families) is the unit of resampling (100 bootstraps used). For each locus, a χ^2 statistic was calculated to test the null hypothesis that the number of observed progeny individuals for each genotype class from each maternal genotype plant did not differ from the expected number under the mixed-mating model. Assumptions of the model are as described in Ritland and Jain (1981). In particular, the model specifies that both selfing and outcrossing occur in the population (Shaw and Allard, 1982).

RESULTS

Thirty seven out of 50 loci assayed had significant differences between the allele frequencies of ovules and incoming pollen at the 95% level (Table 2). This suggests that the maternal trees did not represent all the local pollen pools. However, this violation of the assumption of the equivalence of pollen allele frequencies received by the maternal trees has an unmeasurable but relatively minor effect on the estimate of the true population outcrossing rate (Ritland and Jain, 1981). A χ^2 statistic to test the conformity of marker loci to the mixed-mating model, indicated that for seventeen AFLP markers the number of observed progeny individuals for each genotype class from each maternal genotype departed from the expected numbers (Table 2).

Table 1. Sequences of the AFLP primers and the corresponding adapters used in this study.

Primer	Nomenclature	Sequence
*Pst*I adaptors Forward		5′-CTCGTAGACTGCGTACATGCA-3′
Reverse		5’-TGTACGCAGTCTAC-3′
*Pst*I Primers	POO	5′-GAC TGC GTA CAT GCA G-3′
	P11	POO + AA
	P12	POO + AC
*Mse*I adaptors Forward		5′-GACGATGAGTCCTGAG-3′
Reverse		5′-TACTCAGGACTCAT-3′
*Mse*I Primers	MOO	5′-GAT GAG TCC TGA GTA A-3′
	M51	MOO + CCA

Table 2. Allele frequencies, their respective standard deviations (σ), χ^2 statistics for agreement with the mixed-mating model.

Gene frequency				Gene frequency			
Locus	**Pollen (σ)**	**Ovule**	**χ^2**	**Locus**	**Pollen(σ)**	**ovule**	**χ^2**
P12-1	0.09 (0.07)	0.2	3.36	**P11-26**	0.05 (0.01)	0.5	10.43*
P12-2	0.06 (0.03)	0.1	0.86	**P11-27**	0.05 (0.03)	0.0	0.05
P12-3	0.30 (0.07)	0.1	2.60	**P11-28**	0.06 (0.03)	0.0	0.10
P12-4	0.03 (0.03)	0.1	6.38*	**P11-29**	0.13 (0.04)	0.0	0.04
P12-5	0.04 (0.04)	0.1	13.54*	**P11-30**	0.06 (0.04)	0.1	5.71*
P12-6	0.04 (0.04)	0.1	6.75*	**P11-31**	0.19 (0.06)	0.0	1.22
P12-7	0.08 (0.05)	0.1	10.42*	**P11-32**	0.08 (0.05)	0.1	7.21*
P12-8	0.49 (0.09)	0.2	0.76	**P11-33**	0.18 (0.08)	0.3	1.25
P12-9	0.09 (0.04)	0.2	0.07	**P11-34**	0.00 (0.00)	0.1	0.58
P12-10	0.17 (0.06)	0.2	2.12	**P11-35**	0.12 (0.05)	0.1	3.02
P12-11	0.21 (0.09)	0.3	6.84*	**P11-36**	0.03 (0.02)	0.0	0.00
P12-12	0.00 (0.00)	0.1	6.37*	**P11-37**	0.06 (0.04)	0.1	10.49*
P12-13	0.00 (0.00)	0.2	2.08	**P11-38**	0.16 (0.06)	0.1	8.55*
P12-14	0.17 (0.07)	0.1	11.18*	**P11-39**	0.02 (0.02)	0.0	0.01
P12-15	0.01 (0.00)	0.5	2.76	**P11-40**	0.02 (0.02)	0.0	0.01
P12-16	0.07 (0.04)	0.4	0.07	**P11-41**	0.19 (0.04)	0.0	0.16
P12-17	0.02 (0.00)	0.5	9.01*	**P11-42**	0.20 (0.08)	0.3	3.93*
P12-18	0.04 (0.03)	0.4	0.02	**P11-43**	0.98 (0.03)	0.9	0.02
P12-19	0.03 (0.00)	0.5	11.85*	**P11-44**	0.65 (0.11)	0.8	0.05
P12-20	0.01 (0.00)	0.5	0.10	**P11-45**	0.02 (0.02)	0.2	0.00
P12-21	0.77 (0.07)	0.9	0.00	**P11-46**	0.02 (0.02)	0.2	0.00
P12-22	0.02 (0.02)	0.0	0.00	**P11-47**	0.02 (0.02)	0.2	0.00
P12-23	0.02 (0.02)	0.0	0.00	**P11-48**	0.02 (0.02)	0.2	0.00
P12-24	0.06 (0.02)	0.5	13.03*	**P11-49**	0.02 (0.02)	0.2	0.00
P12-25	0.05 (0.01)	0.5	10.43*	**P11-50**	0.18 (0.08)	0.8	0.04

*****Marker locus with significant deviation at the 0.05 level.
Standard error for paternal gene frequencies were not computed because sampling was done within families.

The estimates of multilocus outcrossing rates (t_m) and single-locus outcrossing rates (t_s) obtained from MLDT clearly indicate self compatibility in the *M. oleifera* mating system (Table 3). The multilocus outcrossing rate estimates based on all the 50 loci was 74%. Selfing may not be the only form of inbreeding experienced by a plant population. To investigate the possibility of biparental inbreeding (inbreeding arising from mating among related plants), the difference between the multilocus outcrossing rate estimates and the mean of the single locus estimate

Table 3. Multilocus (t_m) and single-locus (t_s) outcrossing rates and Wrights fixation index (*F*) (standard errors in parentheses).

Site	Families	Offspring	t_m	t_s	t_m-t_s	*F*
Mbololo	4	86	0.740 (0.065)	0.522 (0.050)	0.219 (0.043)	0.376 (0.000)*

*Standard error for *F* was not computed because sampling was done within families.

was calculated (Table 3). There was significant difference between multilocus estimates and the mean of the single locus estimates, suggesting the existence of mating among relatives (biparental inbreeding).

DISCUSSION

The present work demonstrates that AFLP markers, though dominant with a lower information content than co-dominant markers are adequate for the study of the mating system in M. oleifera. The estimates of outcrossing rates obtained indicate that *M. oleifera* seeds from the Mbololo seed source are a product of both selfing and outcrossing events. The mixed mating system (t_m = 0.74) described for this species is consistent with the observations of self-compatibility in India (Puri, 1941; Jyoth et al., 1990). Comparable levels of outcrossing have been observed in some species such as *Schiedea lydgatei* (t_m =0.694-0.874), *Hydrophyllum appendiculatum* (t_m=0.62-0.81) by Norman et al. (1997) and Wolfe and Shore (1992), respectively. However, high outcrossing rates (t_m > 0.9) have been observed in a majority of conifers (Furnier and Adams, 1986; Morgante et al., 1991; Cottrell and White, 1995).

Multilocus estimation is statistically more efficient than single-locus estimation because multilocus data sets contain more information about outcrossing than is available at any one single locus (Furnier and Adams, 1986). Single-locus estimation is more sensitive to related matings other than selfing (Furnier and Adams, 1986). Thus, if inbreeding other than selfing occurs, t_s will generally underestimate outcrossing to a much greater extent than t_m. In the current work, multilocus estimate differed significantly from the single-locus estimate, suggesting significant biparental inbreeding (Ritland, 1990). Possible reasons for significant differences between the allele frequencies of ovules and incoming pollen (Table 3) have been advocated (Murawski and Hamrick, 1992; Furnier and Adams, 1986). Of these, the immigrant pollen from outside the sample population or from an unrepresented sample of maternal trees due to the small number of families sampled may account for the significant differences detected between the allele frequencies of ovules and incoming pollen observed in the present work.

The fixation index, *F*, in the progeny was higher than expected based on the estimate of t_m. Taking t_m = 0.740, the expected fixation index was [$F = (1-t)/(1+t)$] = 0.149, while the estimated *F* was 0.376. A higher than expected *F* suggests more inbreeding than expected in the progeny population used to carry out the study. Since the mating system in *M. oleifera* involves some selfing, an excess of homozygotes in progenies would be expected if the populations are in mating-system equilibrium (Furnier and Adams, 1986). Mating-system studies of natural populations of *Eucalyptus*, reported an *F* higher than expected based on the estimated t_m (Peters et al., 1990; House and Bell, 1994). A χ^2 test indicated that observed progeny genotype frequencies did not conform to those expected under mixed mating for some marker loci. Several factors can contribute to such violations: selection against homozygous genotypes, genotype-dependent outcrossing rate, and unbalanced frequencies of pollen in the population (Ritland, 1983).

In estimating heritability and genetic gains, the assumption that the relationships among the progeny is 0.25 leads to inaccurate estimation of the additive variance if the relationships among the progeny are not entirely half sib (Falconer, 1960; Mousseau and Roff, 1987; Askew and El-Kassaby, 1994). Therefore, a 26% selfing in *M. oleifera* can result in an increase of the coefficient of relationship which can lead to overestimation of the proportion of additive genetic variance. According to El-Kassaby et al. (1994), great overestimation will be achieved if selfing is ignored because selfing contributes additional factors to the covariance between relatives, including dominance and inbreeding depression effects and appropriate adjustments to estimators of quantitative genetic parameters are required.

The presence of selfing as well as early sexual maturity (6 months to 1 year) might provide an alternative breeding programme for this species as compared with traditional directional selection. If inbreeding depression is weak, then it should be possible to use breeding schemes involving inbred lines and hybridisation (El-Kassaby et al., 1994). In designing seed orchards, randomisation and minimum distance between related individuals will need to be worked out to maximise cross-fertilisation among unrelated clones and to minimise selfing or mating among related ramets. The above suggestions are further strengthened by previous observations in India where hand-pollination with xenogamous pollen gave 100% fruit set, 81% seed set and 9% fecundity, while with geitonogamous pollen the respective rates were 62, 64 and 6% (Jyoth, 1990). Future studies should focus on outcrossing rates of

individuals and populations in relation to mechanisms (or environmental parameters) that favour either outcrossing or selfing.

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