Euphytica **138:** 205–212, 2004.

In situ estimation of outcrossing rate in sorghum landraces using microsatellite markers

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Received 11 November 2003; accepted 21 June 2004

Key words: landrace, mating system, microsatellite, outcrossing rate, *Sorghum bicolor*

Summary

We assessed the outcrossing rate of sorghum landraces sampled *in situ* from two fields under traditional cultivation in north-western Morocco using genotypic data from five microsatellite loci. Assuming a mixed mating model, we estimated outcrossing parameters by two methods that are based on progeny analyses. With both methods, the multilocus estimate of outcrossing rate for the overall sample was in the order of 0.1, meaning that sorghum landraces are predominantly autogamous, but with a significant proportion of outcrossing. The estimated outcrossing rate is about two times higher in field 1 ($t_m = 0.161$) than in field 2 ($t_m = 0.069$). This difference could be explained by distinct morphological characteristics of the inflorescence in the two fields, with predominance of loose panicles in field 1 and of very compact panicles in field 2. The distribution of outcrossing rate among progeny families showed that 30% of them were completely self-fertilized but some families showed substantial outcrossing. These results are at odds with the very low genetic differentiation observed previously among Moroccan landraces and suggest that morphological differences are maintained despite gene flow through seed exchanges among farmers.

Introduction

The mating system is one of the most important life history traits of a plant species. It has a large impact on plant population genetic structure, with selfing species showing on average lower genetic diversity within and higher diversity among populations, as compared to outcrossing species (Hamrick & Godt, 1990). Assessment of the mating system for a crop is particularly important for the design of adequate breeding and genetic conservation programs. In the context of an increasing interest in *in situ* conservation approaches focusing on genetic resources of domesticated plants, knowledge of their level of outcrossing under current field conditions is highly desirable. For example, Wells et al. (1988) observed in common bean, a predominantly selfing crop, an important variation in outcrossing rate among six lines obtained through recurrent selection.

Cultivated sorghum (*Sorghum bicolor* (L.) Moench) is an important food crop in the world, cultivated mainly in Africa and Asia. Sorghum is an annual and wind-pollinated cereal that is known to be predominantly selfing (Chantereau & Nicou, 1991; Doggett, 1988). Under experimental conditions, Ellstrand & Foster (1983) showed that the outcrossing rate (t) was influenced by population structure and obtained an average value of $t = 0.30$. Ollitrault et al. (1997) found a mean value of $t = 0.19$ for

sorghum landraces from Burkina-Faso belonging to the guinea race, based on progeny analysis. Using an indirect method based on the value of the inbreeding coefficient, we previously reported a mean value of $t = 0.18$ under field conditions in landraces from north-western Morocco grown according to traditional practices (Djè et al., 1999). Although this value was in agreement with earlier estimates of the outcrossing rate in sorghum, it seemed to contradict the very low levels of genetic differentiation observed among fields in the Moroccan landraces ($G_{ST} \approx 0.1$ in Djè et al. (1999); $G_{ST} = 0.17$ in Vekemans et al. (2001)), which lie in a range that is common for outcrossing rather than selfing species (Hamrick & Godt, 1990).

Direct estimation methods of the outcrossing rate are based on the mixed mating model, which assumes that every individual in the population produces a proportion s of its seeds through self-fertilization and a proportion $t = 1 - s$ through cross-fertilization with pollen randomly dispersed from other plants (Clegg, 1980). Seeds from several mother plants sampled in the field are analyzed at genetic marker loci and the genotypes of mother and offspring are compared to estimate the outcrossing rate using either an iterative maximum likelihood approach (Ritland & Jain, 1981; Ritland, 2002) or a method based on a direct count of detectable outcrossing events (Shaw et al., 1981). Traditionally, morphological or allozyme markers have been used for this purpose (Godt & Hamrick, 1991; Holtsford & Ellstrand, 1989), but more recently, molecular markers targeting DNA sequences represent a new alternative for mating system estimations (Gaiotto et al., 1997; Streiff et al., 1999) because of their high polymorphism. In a study of sorghum landraces from north-western Morocco (Djè et al., 1999), we found that the average proportion of heterozygote individuals was much lower for allozyme ($H_o = 0.012$) than for microsatellite loci ($H_o = 0.272$), and pointed out that microsatellites would thus be the more appropriate tool for direct estimation of mating system parameters in the field. The aim of this study is to highlight the potential of microsatellites as genetic markers to study crop mating systems under field conditions. We estimated outcrossing rate in two fields of sorghum landraces from north-western Morocco using a progeny analysis technique based on genotypic data at five microsatellite loci. The two fields were chosen to represent two different morphological types of sorghum landraces from Morocco in order to detect potential differences in mating system in relation to morphological characteristics.

Materials and methods

Plant material and morphological analysis

According to the classification of Harlan & de Wet (1972), sorghum landraces cultivated in north-western Morocco belong to races durra and the intermediate durra-bicolor (Kadiri & Ater, 1997). Twenty-five panicles were randomly harvested in each of two fields of sorghum from north-western Morocco (Figure 1). Field 1 is located approximately 30 km south-east of

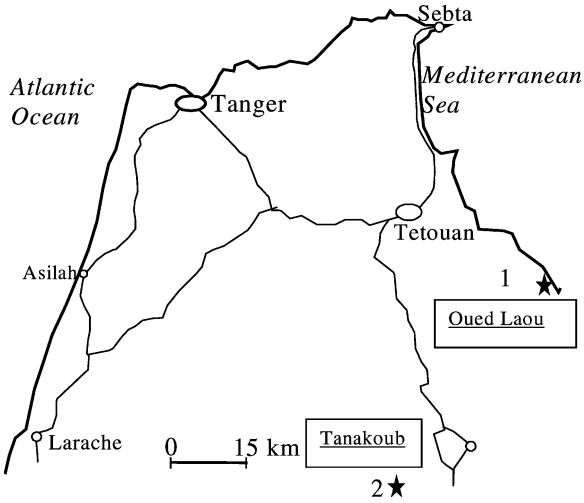

Figure 1. Location of the sorghum fields sampled from Morocco.

Tetouan at the Mediterranean sea level, on alluvial rich deposits of the Oued Laou, and was chosen because the plants are morphologically of the intermediate type durra-bicolor. Field 2 is located inland, 70 km southwest of Tetouan on mineral soil at 600 m altitude, and the plants present the morphological type of the race durra. In order to evaluate morphological differences between the two fields, the following characteristics of the inflorescence were measured on each panicle: length of the panicle in cm (PAL); largest diameter of the panicle in mm (PAD); weight of the whole panicle in g (PAW); shape of the panicle (PASH, encoded as 1-very loose, 2-loose, 3-compact, and 4-very compact, which correspond respectively to types 2–3, 4, 5 and 6–7 in the classification of Harlan & de Wet (1972)). The proportion of the grain covered by the glumes (GRCOV, encoded as 1–<50% covered, 2–50–75% covered, 3–75–100% covered) was measured as it discriminates between the morphological types bicolor and durra (Harlan & de Wet, 1972).

Extraction of DNA and microsatellite analysis

Eight seeds per individual were germinated in a dark chamber at 22 °C and seedlings were transferred to individual pots into the greenhouse. After 4 weeks, young leaves (100–150 mg) were used for extraction of genomic DNA following a CTAB procedure (Doyle & Doyle, 1990). Five primer pairs of microsatellite loci, which were previously shown to display a high degree of polymorphism in sorghum, were used in amplification reactions (Brown et al., 1996; Taramino et al., 1997). The polymerase chain reactions (PCR) were performed in a mix containing Mg Cl_2 2.5 mM, 0.5 unit per reaction of AmpliTaq Gold™ DNA polymerase in buffer II (Applied Biosystems) and 10 ng template DNA (2.5 μl), in a total reaction volume of 25 μl. PCR cycling conditions were as follows: 10 min initial denaturation at 95 °C, 34 cycles of amplification [30 s at 94 °C, 45 s at either 55 °C (Sb4-22, Sb5-236 and SbAGH-04) or 53 °C (SbAGA-01 and SbAGE-01), 1 min elongation at 72 °C], and final elongation of 5 min at 72 °C. PCR reactions were performed on a Mastercycler® Gradient 2331 version 1.2 (Eppendorf). The forward sequence of each primer pair was labelled with a fluorescent dye (Applied Biosystems) at its 5′ end: 6-FAM for Sb5-236 and Sb4-22; HEX for SbAGA-01 and SbAGE-01; and NED for SbAGH-04. The dyes were chosen in order to distinguish the different loci among PCR-products of similar sizes. This allowed the pooling of PCR-products from all loci in each well, after appropriate adjustment of their relative concentration. An internal size standard (Genescan-350 with ROX dye) was also loaded in each well. Electrophoresis and detection of PCR products were carried out on denaturing polyacrylamide gels (5% Long Ranger; 36 cm-length) using an ABI PRISM® 377 DNA sequencer from Applied Biosystems with filter set D. Gels were run during 2 h at 3000 V in TBE buffer. Resulting electrophoregrams were analyzed with the software Genescan® 2.0 (Applied Biosystems).

Data analysis

Student's t-tests were performed on the morphological data, after checking for departure from normal distribution and for unequal variances, with the software Statistica v. 6.0.

Analyses of genotypic data were performed using the computer program GEN-SURVEY (Vekemans & Lefèbvre, 1997). Genetic variation within each field was estimated using the mean number of alleles per locus, A, and the average gene diversity, H_e, computed according to Nei (1978). The genetic differentiation between the two field samples was estimated from Wright's F_{ST}-statistic computed according to Weir & Cockerham (1984).

Estimation of the outcrossing rate was performed using two methods based on the mixed mating model, with assessment of the genotype of the maternal plants according to Brown & Allard (1970). (1) The iterative maximum likelihood procedure of Ritland & Jain (1981) uses a Newton-Raphson-based algorithm of iteration and is computed with the program MLTR (Ritland, 1990; Ritland, 2002). It produced estimators of the multilocus outcrossing rate (t_m) and of the single locus outcrossing rate (t_s) averaged across loci. Estimators of the multilocus outcrossing rate for each maternal plant were also computed. Standard errors for these statistics were calculated from 500 bootstraps estimated by resampling among progeny families. (2) The second method proceeds by a direct count of the number of detectable outcrossing events, occurring when non-maternal alleles are found in a progeny, followed by a statistical correction for the outcrossing events remaining undetected (Shaw et al., 1981). The value of the outcrossing rate (t) is computed as:

$$t_m = \frac{n}{N(1-\alpha)}$$

where N represents the total number of offspring, n is the number of offspring for which a non-maternal allele was found in at least one locus, and α is the probability of misidentification of outcrossed progeny, which can be estimated as:

$$\alpha = \prod_{k=1}^{m} \left[\sum_{f} \frac{ndesc_f}{N} (p_{ik} + p_{jk}) \right]$$

where m is the number of loci, $ndesc_f$ is the size of the progeny array analyzed from maternal individual f, p_{ik} and p_{jk} are the frequencies of the two maternal alleles i and j at locus k in the pollen pool (if the mother f is homozygous for allele i, $(p_{ik} + p_{jk})$ reduces to p_{ik}).

Results

Morphological analysis

The two fields are shown to differ significantly for all morphological characters except the weight of the panicle (PAW; Table 1). Plants from field 1 had on average longer and wider panicles than those from field 2, which suggests that inflorescences are more compact in field 2 than in field 1 because their average weights are similar. This was confirmed by the estimation of the shape of the panicle (PASH) which was "very loose" to "compact" in field 1, but predominantly "very compact" in field 2, according to the classification of Harlan & de Wet (1972). The proportion of the grain covered by the glumes was significantly higher in plants from field 1 than from field 2.

Number of alleles and genetic diversity

The numbers of alleles observed at each locus in each field and in the overall sample are given in Table 2. Allelic richness was quite high with four out of five loci showing more than 10 alleles in the overall sample. Several alleles were present in one field but absent from the other. The numbers of alleles observed in the two fields were substantially lower than in a sample consisting of 25 accessions from the world sorghum genebank (Djè et al., 2000). This was especially true for locus Sb4-22 where only 4 out of 14 (29%) alleles were observed in the sample from Morocco.

Levels of genetic variation within both fields were very similar: the mean numbers of alleles per locus (A) are 8.4 and 8.0; and the average gene diversity (H_e)

Table 1. Morphological characteristics of panicles from the two fields of sorghum

Field	PAL (cm)	PAD (mm)	PAW (g)	PASH	GRCOV
1	15.9 ± 2.4	48.8 ± 11.2	18.39 ± 8.60	2.16 ± 0.85	1.80 ± 0.41
2	10.4 ± 3.5	41.2 ± 8.7	19.68 ± 11.1	3.48 ± 0.87	1.36 ± 0.49
Student's t-test	$P < 0.0001$	$P < 0.05$	Not significant	$P < 0.0001$	$P < 0.01$

Means are given ± standard deviation. PAL, panicle length in cm; PAD, panicle largest diameter in mm; PAW, panicle weight in g; PASH, index of panicle shape (see text); GRCOV, index of grain coverage by the glumes.

Table 2. Type of repeat and number of alleles observed at each microsatellite locus

		Number of alleles observed			
Locus	Repeat motif	Field 1	Field 2	Overall sample	Genebank[a]
SbAGA01[b]	$(AG)_{33}$	8	9	11	18
SbAGH04[b]	$(AG)_{39}$	11	6	11	24
Sb5-236[c]	$(AG)_{20}$	10	11	15	17
SbAGE01[b]	$(AG)_{30}$	9	12	12	23
Sb4-22[c]	$(ACGAC)_4/(AG)_6$	4	2	4	14

[a]Number of alleles observed in a sample of 25 accessions from the world genebank (Djè et al., 2000).
[b]From Taramino et al. (1997).
[c]From Brown et al. (1996).

Table 3. Mean values of multilocus (t_m) and single locus (t_s) estimates of outcrossing rate within sorghum landraces

	Method of Ritland & Jain (1981)				Method of Shaw et al. (1981)	
	Field 1		Field 2		Field 1	Field 2
Locus	Mean t[a]	S.D.	Mean t[a]	S.D.	Contribution to α	Contribution to α
SbAGA01[b]	0.060	0.023	0.042	0.019	0.256	0.335
SbAGH04[b]	0.047	0.044	0.064	0.021	0.248	0.274
Sb5-236[c]	0.099	0.028	0.038	0.014	0.278	0.216
SbAGE01[b]	0.077	0.032	0.035	0.012	0.228	0.232
Sb4-22[c]	0.150	0.071	0.051	0.028	0.608	0.659
Average t_s	0.088	0.025	0.043	0.013		
t_m	0.161	0.038	0.069	0.017	0.175	0.064

SD: standard deviation obtained by bootstrapping.
[a]t is the outcrossing rate.
[b]From Taramino et al. (1997).
[c]From Brown et al. (1996).

was 0.743 and 0.739 in fields 1 and 2, respectively. The genetic differentiation for microsatellite loci between the two fields was low ($F_{ST} = 0.02$) despite substantial morphological differences.

Estimation of outcrossing rate

Estimates of the outcrossing rate using the method of Ritland & Jain (1981) are given in Table 3. Values of the outcrossing rate were consistently higher within field 1 than field 2 ($t_m = 0.161 \pm 0.038$ and 0.069 ± 0.017, respectively). These values indicate that 16.1 and 6.9% of the progeny from fields 1 and 2, respectively, were the result of outcrossing events. The mean value of the multilocus estimate of outcrossing rate in the overall sample was $t_m = 0.109 \pm 0.024$. Outcrossing rate estimates were found to vary strikingly among progeny families within each field (Figure 2), with no outcrossing event detected in 28% (14/50) of the families and more than 20% of outcrossing in 24% (12/50) of the families. The level of biparental inbreeding can be estimated as the difference between multilocus and single locus estimates of outcrossing rate ($t_m - t_s$). A higher

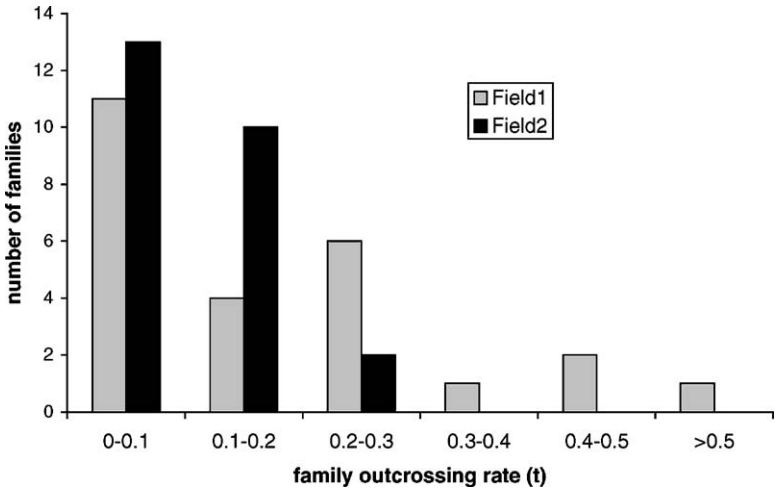

Figure 2. Distributions of the estimate of outcrossing rate among progeny families sampled from two fields of sorghum.

value of $t_m - t_s$ was obtained in field 1 (0.072) when compared to field 2 (0.026).

The number of offspring displaying at least one non-maternal allele was higher within field 1 (35/200) than within field 2 (13/205). The relative contributions of each locus to the expected proportion of undetected outcrossing events α (Shaw et al., 1981) are given in Table 3. It appears that locus Sb4-22 contributes to a higher extent to α than the other loci owing to its lower number of alleles. The value of α was very low ($\alpha = 0.002$ and 0.003 in fields 1 and 2, respectively), indicating that a very high proportion of outcrossing events were detected. Estimates of the outcrossing rate according to the formula of Shaw et al. (1981) were $t_m = 0.175$ and 0.064 for fields 1 and 2, respectively, values which are remarkably close to those derived from the method of Ritland & Jain (1981).

Discussion

The allelic richness observed within the two fields ($A = 8.4, 8.0$) is higher than that found in our previous study on Moroccan sorghum field populations genotyped with three microsatellites ($A = 6$, Djè et al., 1999). As the sample size for fields was similar in both studies, we suggest that the actual detection of a higher number of alleles per locus is explained by using a high resolution electrophoresis technique coupled with a fluorescent detection system, as opposed to classical electrophoresis on short poly-acrylamide gels with ethidium bromide staining. Other parameters of genetic variation (H_e) agreed with earlier results. Overall this confirms our previous conclusions, that sorghum landraces from north-western Morocco are characterized by a high level of genetic diversity within individual fields. A field thus constitutes a valuable unit of conservation in itself and this has practical consequences in designing *in situ* conservation programs for sorghum (Djè et al., 1999).

Genetic differentiation between the two fields, as estimated from microsatellite loci was very low. This contrasts with the strong morphological differences that were observed between the landraces cultivated in the two fields. Plants from field 1 had open and loose panicles and the grains were largely covered by the glumes, two characteristics that are typical of an intermediate morphological type durra-bicolor (Harlan & de Wet, 1972). Plants from field 2 were morphologically typical of the race durra, with very compact panicles and grains that were only partially covered by the glumes. Such discrepancy between morphological classification and differentiation based on neutral genetic markers has been pointed out by Morden et al. (1989) who showed that genetic variation was more closely associated with geographic origin than racial classification, and was further confirmed by our previous survey of microsatellite variation within and among worldwide accessions belonging to different morphological races (Djè et al., 2000).

Two different methods were used to estimate the outcrossing rate. Both methods showed that the outcrossing rate was about two times higher within field 1 than field 2, and the estimates from both methods are remarkably similar. The consistency between the results from both methods may be due to the large number of alleles at the microsatellite loci, which increases the reliability of the method of Shaw et al. (1981). Indeed we observed that with the five microsatellite loci, the probability of not detecting an outcrossing event was very low ($\alpha < 0.005$). The estimate of the mean outcrossing rate was about 16% in field 1 but only about 7% in field 2. These values lie in the range of outcrossing rates typically reported in sorghum under experimental conditions (Doggett (1988), $t = 0.05$; Ellstrand & Foster (1983), $t = 0.30$; Ollitrault et al. (1997), $t = 0.19$). The estimate from field 1 is also in agreement with our previous average estimate of $t = 18\%$ in Moroccan sorghum landraces computed from observed values of the inbreeding coefficient (Djè et al., 1999). Hence, we can conclude that sorghum landraces from north-western Morocco are predominantly autogamous. The very low differentiation observed among fields at neutral genetic markers ($G_{ST} \approx 0.1$, Djè et al. (1999); $G_{ST} = 0.17$, Vekemans et al. (2001); $F_{ST} = 0.02$, this paper) remains thus in contradiction with the mating system and with the differentiation for morphological characters (63% of variation among fields; Djè et al., 1998). We suggest that the low neutral genetic differentiation among fields and among regions reported previously is caused by: (1) recurrent seed exchanges among farmers; (2) an occasional seed supply from local public markets after accidental harvest losses; and (3) the relatively recent introduction of the sorghum crop in Morocco (Kadiri & Ater, 1997). In this context of recurrent gene flow or, alternatively of recent common origin, morphological differences among landraces may have arisen and be maintained as a consequence of phenotypic selection by farmers.

An interesting observation is that the outcrossing rate varied between the two fields and also among

progeny families within fields. These results could be explained by features of inflorescence morphology and variation in flowering phenology. A higher outcrossing rate was observed in field 1 that is characterized by loose panicles, than in field 2, that shows more compact panicles. Hence, we suggest that the shape of the panicles in sorghum influences the mating system, with the architecture of very compact panicles impeding pollination with outcrossed pollen. Because ancestral sorghum types had loose panicles (Harlan & de Wet, 1972) we further hypothesize that domestication of sorghum has been accompanied by an increase in the selfing rate. Within fields, large variation in the delay between the maturity of anthers and stigmas has been reported in sorghum by Pendleton et al. (1994). Such phenomenon could cause differences in outcrossing rate among plants. Another possible explanation has been put forward by Maunder & Sharp (1963) and Ollitrault (1987) who reported different values of outcrossing rate in relation to spikelet position in the panicle within sorghum landraces: seeds taken from the top of the panicle exhibited somewhat higher outcrossing values than seeds taken from the lower part of the panicle. Doggett (1988) also reported that the form of the peduncle (curved or straight) could have a great influence on outcrossing.

In conclusion, our results based on seed samples taken *in situ* indicated that in cultivation sorghum is predominantly selfing, as was previously suggested from studies under experimental conditions. Differences in selfing rate between the two fields investigated could be explained by distinct morphological characteristics of the inflorescence. The autogamous mating system is at odds with the very low genetic differentiation observed previously among Moroccan landraces and this suggests that gene flow through seed exchanges among farmers is frequently occurring, with morphological differences among landraces maintained by phenotypic selection by farmers.

Acknowledgments

We thank K. Ritland for sending a new version of his MLTR program and M. Milinkovitch for allowing use of his laboratory equipment. This study has been granted by the "Université Libre de Bruxelles" (Belgium) and the "Université Abdelmalek Essaadi-Tétouan" (Morocco) in the framework of bilateral scientific collaboration.

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