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Estimation of Outcrossing Rate in Ethiopian Sesame (*Sesamum indicum* L.) using SSR Markers

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Abstract: **Sesame (*Sesamum indicum* L.) is generally considered to be a self-pollinated crop. Information about the out-crossing rate of Ethiopian sesame is not known. The aim of this study was to obtain reliable estimates of the amount and variation of out-crossing in Ethiopian sesame. Natural out-crossing was estimated in 47 sesame landraces representing the existing homeland collections and 3 released sesame varieties. Ten PCR based molecular markers, Simple Sequence Repeats (SSRs) have been used to estimate the out-crossing percentage based on the equilibrium inbreeding coefficient (F_e) under partial selfing. The Polymorphism Information Contents (PICs) of the markers ranged from 0.393 to 0.820, which indicates the highly informative nature of the SSRs reported here. The observed heterozygosity (H_o) for landraces and cultivars ranged from 0.060 to 0.440 and 0.120 to 0.260, with a mean of 0.234 and 0.193, respectively, indicating the existence of higher heterozygosity within the landraces than cultivars. Landraces also showed higher diversity compared to the cultivars in terms of Nei's average genetic diversity of 0.377 compared with 0.305, respectively. High outcrossing of 45% and 46.4% have been estimated for landraces and cultivars, respectively, indicating both landraces and cultivars are not lines but line mixtures and segregants of past out-crossing results. Existence of high out-crossing rate among the landraces is a good starting point for sesame breeding, germplasm management and conservation in Ethiopia.**

Keywords: **Matting system, Molecular markers; Out-crossing, Sesame, *Sesamum indicum* L, SSR.**

1. INTRODUCTION

Flowering plants produce offspring through different types of mating systems. This variation is a consequence of distinctive features of their biology. The plant mating system plays a fundamental role in determining the spatial and temporal patterns of genetic diversity within and between populations and hence their evolutionary dynamics. Plant breeders have recognized the importance of mating system for breeding, germplasm management and conservation. The proportion of selfing to out-crossing influences formulation of optimal strategies for hybridization, recombination of genotypes, genetic sampling and the way germplasm collection should be maintained *ex situ* and *in situ*. The amount of out-crossing is a crucial characteristic in the exploitation of hetrosis through synthetic cultivars and for seed multiplication, and variety maintenance (Barrett, 2003; Dje et al., 2004; Freitas, 2004; Spencer, 2010).

Outcrossing rate in plants is genetically controlled but influenced by different factors. Flower morphology and color, rainfall, temperature, light intensity and elevation (Ritland,1983; Abdel Ghani et al., 2004; Parzies et al., 2008), the diversity, abundance, activity and behavior of pollinating agents and plant population density can influence the outcrossing of a plant species (Krueger and Knapp, 1991; Suso et al., 2001). Therefore, knowledge of the outcrossing rate of a crop species in a specific environment is important. Studies to determine outcrossing rate have usually been conducted using easily identifiable morphological markers, but the development of biochemical and molecular markers

paved the way for easier and reliable techniques (Gaiotto et al.,1997; Muluvi et al., 2004). Molecular markers based on DNA sequences are more reliable than unstable morphological markers, hence can be used to estimate the out-crossing rate of crops. Microsatellites, also called simple sequence repeat (SSR) markers, are clusters of short tandemly repeated nucleotide bases spread throughout the genomes of all eukaryotes. They are characterized by high variability, co-dominant nature, great abundance and even distribution throughout a wide range of genomic regions (Tautz, 1989, Ivandic et al., 2002). Microsatellites have been employed successfully in barley for estimation of outcrossing rates and in genetic diversity studies (Maestri et al., 2002; Abdel-Ghani et al., 2004, Brantestam et al., 2007, Parzies et al., 2008; Jilal et al., 2008). Sesame (*Sesamum indicum* L.) is generally regarded as a self pollinated crop (Weiss, 1983; Ashir, 1998). Ethiopian Breeders considered sesame as a predominantly self pollinated crop. Sesame improvement in Ethiopia has been mostly done by employing breeding methodologies used for self-fertilized crops (Anonymous, 2007). So far, there is no report that describes the level of out crossing rate in sesame using molecular markers. The objective of this work was to estimate out-crossing rate of sesame using microsatellite markers.

2. MATERIALS AND METHODS

2.1 Plant materials:

Seeds of 47 landraces of sesame collected in Ethiopia and three nationally released cultivars named: K74, M80 and Tate were obtained from Ethiopian Institute of Agricultural Research (EIAR), Werer Agricultural Research Center. They are listed in Table 2 with information regarding their origin. The landraces were collected from different regions covering sesame growing areas in Ethiopia. Five seeds from each population (landraces/cultivars) were planted separately in a soil in a 77 cell tray in a green house at Hohenheim University, Germany. A total of 250 seedlings were obtained. Four weeks old plants were transplanted to pots and arranged per landrace/cultivar in the green house. Young leaves (2-3 leaves) free of any infestation were collected and dried in filter paper bags on the silica gel for 48 hours.

2.2 DNA extraction:

Genomic DNA was extracted from dried leaf tissues employing a 3% CTAB procedure (Saghai Maroof et al., 1984) with modification. Dried leaf materials were cut in to small pieces and placed in labeled deep well racks. One tungsten carbide bead was placed in each deep well rack along with the dried leaf material and the deep well racks were covered with plastic caps and grinded using a tissuelyser (2 x 5 min) until the leaf material was homogenized. Pre- heated (65 °C) 3 % CTBA (600µl) buffer was added into each deep well racks and vortexed until the powder was dissolved. The mixture was centrifuged at 4000 rpm for 1 min. The cover caps were removed from each deep well racks and covered with a plastic cover and incubated in a water bath at 65 °C for 45 min with occasional shaking and then cooled to room temperature for 15 min. The mixture was centrifuged again at 4000 rpm for 1 min and Chloroform/ Isoamylalcohol (24:1, vol:vol, 400 µl) was added then the solution was mixed gently by hand shake for 3-4 min and centrifuged at 3500 rpm for 45 min. The supernatant was decanted into new autoclaved and labeled Eppendorf tubes and 800µl ice cold Isopropanol was added and inverted gently for a few times (4-5 min). The mixture was centrifuged at 3000 rpm for 3 min. After pouring off the top aqueous solution precipitated DNA was found as pellets at the bottom of the Eppendorf tubes. The pellet was washed with 800 µl of wash buffer (380 ml alcohol obsolute, 5ml 1M ammonium acetate and water) and incubated in the water bath at 40 °C for 20 min. Then the mixture was centrifuged at 3000 rpm for 1 min. The wash buffer was removed from each Eppendorf tube with great care not to lose the DNA pellet. DNA pellets were dried in the laminar air flow cabinet for 3hrs. Then 1x TE buffer (100 µl) was added to each Eppendorf tube to dissolve the dried pellets. Rnase (3 µl) was added to the DNA solution to catalyze the hydrolysis of ribonucleic acid and incubated in the water bath at 37 °C for 30 min. DNA samples were stored at – 20 °C in TE buffer until measurement of concentration.

2.3 Determination of DNA quality and concentration:

In order to check quality and concentration, DNA was subjected to electrophoresis on 0.8 % agarose gels. For this agarose (1.6 g) was dissolved in 200 ml 1 x TBE buffer by permanent mixing using a magnetic stirrer. Uncut lambda DNA standards at concentrations of 10 and 50 µl were included on gel to estimate the DNA concentration. DNA sample mixtures (2µl DNA, 4µl H_20 and 2µl of bromophenol blue) and Lamda- DNA standards were pipetted in to wells of the submerged gels in an electrophoresis unit containing 1 xTBE buffer. An electric current of 140 V was applied for 45 min. Then the gel was stained in Ethidium Bromide (5µg/ml) for 10 min and distained in distilled water for 15 min. DNA was

visualized using ultraviolet light and photographed using a video capture system (Flowgen IS 1000). The concentration of the DNA fragments was estimated comparing with uncut lambda DNA and DNA samples were diluted accordingly.

2.4. Polymerase chain reaction conditions and amplifications:

PCR conditions were maintained and each PCR reaction was carried out in a 10µl reaction volume containing bidest water, 1.5 mM MgCl2, 1 x 1.5 mM Mgcl2 of 10 x PCR buffer, 0.2 mM of deoxyribonucleotides, 250 nM of each forward and reverse primers, 0.5 units of Taq DNA polymerase and 10ng of DNA. The temperature profile used for PCR amplification comprised a denaturation step at 94 °C for 1 min, followed by primer annealing temperature at 45.2 -53 °C for 1 min, and elongation at 72 °C for 1 min. After 34 cycles, the reaction was ended up with 10 min at 72 °C for the final extension. The PCR reaction was carried out under the same conditions for all the primers except for the annealing temperatures. The PCR products were electrophoresed on 3% Metaphor AGAROSE Gel (9 gm of metaphor and 300ml of 1xTBE) at 140 V for 2h, DNA ladder (50bp) of 4µl was used for comparison. The gels were stained consequently with ethidium bromide (5µg/ml) for 10 min, de-stained for 15 min in distilled water and then frequently visualized under a UV transilluminator and the picture captured with a digital camera.

2.5. Capillary electrophoresis:

A MegaBACE sequencer (Amersham Biosciences) was used to separate PCR amplification products. Individual PCR products were pooled (1-3 markers/run) according to their fluorescent label to permit discrimination of the individual markers after electrophoretic separation. A 96 well PCR plate format and transfers using eight channel pipettes were used throughout. The internal size standard was labeled with the ET-ROX (ET 400-R, Amersham biosciences). The final cocktail of the samples contain 0.5 µl of multiplexed PCR products and 5µl of diluted ET-ROX standard (1:20 dilution with loading solution). Samples were incubated for 1 min at 94 °C and cooled down before quick centrifuging, and then successfully loaded in the MegaBACE and run for approximately 2 hours.

2.6. Statistical analysis:

The fragment sizes were assessed using MegaBACE Fragment Profiler (Amersham Biosciences) software. The resulting SSR data were analysed statistically using the software 'Tools for Population Genetic Analysis'' (TFPGA) version 1.3 (Miller, 2000) to calculate a distance matrix between pairs of sesame population based on the modified Rogers' distance (MRD) measure (Goodman and Stuber, 1983). The genetic diversity of each SSR marker was measured in terms of number of alleles per locus, allele frequencies, observed heterozygosity (H_o) and Expected heterozygosity (He) also known as Nei's average gene diversity using the "population Genetics "(PopGene) software version of 1.31 (Yeh et al., 1999). The polymorphic information content (PIC) for each marker was determined as described by Anderson et al. (1992):

$$\text{PIC} = 1 - \sum_{i=1}^{n} p_i^2$$ Where p_i^2 is the frequency of i^{th} allele.

The outcrossing rate was estimated based on the equilibrium inbreeding coefficient (F_e) under partial selfing: $F_e = \frac{1-t}{1+t}$ (Crow and Kimura, 1970). This equation can be transformed to:

$$t = \frac{1-F_e}{1+F_e}$$

F_e equals 1 - $\frac{H_e}{H_r}$, where H_e is the equilibrium heterozygosity at a given locus, and H_r the heterozygosity expected under random mating (Hardy –Weinberg Equilibrium). For estimating the outcrossing rate, H_e was replaced by the observed heterozygosity, and H_r replaced by Nei's average gene diversity which equals the expected heterozygosity and the assumption of Hardy-Weinberg Equilibrium. The resulting estimate is designated outcrossing of inbreeding equilibrium t_e.

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3. RESULTS AND DISCUSSIONS

3.1. Levels of polymorphism for SSR markers:

Screening publicly available ESTs data base for sesame resulted in the identification of 2 SSRs. Eight additional SSRs were selected from literature. The two SSRs developed from publicly available ESTs database together with the 8 SSRs selected from the literature are listed in Table 1 with their characteristics. All SSR markers showed polymorphism. Overall, 121 alleles were detected using the 10 SSR markers (Table 1). The number of alleles per SSR marker ranged from 6 to 17 with a mean of 12.1 alleles per marker (Table 1). Eight markers revealed more than 10 alleles and the remaining two markers revealed less than 10 alleles, GBssr-sa-08 (NA=6) and GBssr-sa-108 (NA=9) (Table 1). A wide range of fragment sizes was observed from 135 bp to 371 bp. The observed heterozygosity (H_o) ranged from 0.044 to 0.608, with an average value of 0.232. The PIC values for each marker varied considerably, ranging from 0.393 (GBssr-sa-08) to 0.820 (Si-ssr8920), with a mean of 0.634 (Table 1). The PIC, a measure of allelic diversity at a locus demonstrates the informativeness of the marker. Values of PIC range from 0 to 1 and loci having PIC values near 1 are most desirable (Botstein et al., 1980). Therefore a higher value of PIC would mean greater polymorphism at that locus. In the present study the mean PIC value of the 10 SSRs was 0.634 (Table 1). The high mean PIC value displayed by 10 SSRs markers in Ethiopian sesame population agreed with a previous study (Dixit et al., 2005) on Korean sesame using 10 SSR markers. Higher number of observed heterozygosity and large size of fragment size was recorded compared to the results obtained from the previous study (Dixit et al., 2005) on sesame diversity using SSR markers. Higher heterozygosity in our study indicated existence of higher proportion of individuals in a population that are heterozygous at a given locus compared to the previous study (Dixit et al., 2005), indicating Ethiopian sesame populations are more line mixtures. Several landraces revealed more than one peak during amplifications, which may have resulted from the co-dominant nature of the SSR markers. As reported in a number of genetic-diversity studies on other species and populations (Guohao et al., 2003; Gupta and Varshney, 1999; Charters et al., 1996 and Rongwen et al., 1995), SSR loci were able to uniquely identify each of our Ethiopian sesame populations.

Table 1. Diversity of the 10 SSR markers in the sesame collection from Ethiopia

Locus SSR	GenBank accession name	NA[1]	H_o[2]	PIC[3]	Fragment size range of alleles (bp)
SSR loci from literature					
GBssr-sa-05	AY838904	13	0.564	0.680	259-362
GBssr-sa-08	AY838905	6	0.084	0.393	135-159
GBssr-sa-09	AY838905	13	0.208	0.800	162-257
GBssr-sa-72	AY838905	11	0.384	0.427	205-278
GBssr-sa-108	AY838905	9	0.080	0.682	184-215
GBssr-sa-123	AY838905	12	0.076	0.747	213-314
GBssr-sa-182	AY838905	16	0.224	0.493	207-315
GBssr-sa-184	AY838905	12	0.044	0.661	154-195
ESTs derived SSR loci					
Si-ssr8910	AY838910	12	0.048	0.637	278-371
Si-ssr8920	AY838920	17	0.608	0.820	209-304
Total		121			
Mean		12.1	0.232	0.634	

1= Observed number of alleles; 2= Observed heterozygosity;
3 = Polymorphism information content

3.2. Genetic diversity:

Genetic diversity measures of the 50 sesame populations are presented in Table 2. The average numbers of alleles (Na) per locus were 2.4 and 2.0 for landraces and cultivars, respectively. The average number of alleles ranged from 1.4 to 4.2 for landraces and 1.5 to 2.9 for cultivars, respectively. For the cultivars, Mehado80 (Na= 2.9) and Tate (Na=1.5) showed the highest and lowest allelic richness, respectively. The observed heterozygosity (H_o) for landraces per marker ranged

from 0.060 to 0.440. The average observed heterozygosity for the landraces and cultivars across all 10 loci was 0.234 and 0.193, respectively. The proportion of polymorphic loci per marker ranged from 20 % to 100 % (Table 2). The average percentage of polymorphic loci per marker for landraces was 74.04% and 63.33% for cultivars. Among the 50 population evaluated landrace EW011 is the most diverse with Nei's average gene diversity value of 0.658 and Tejin 0.106 was the least diverse. The landraces are more diverse than the cultivars. The Nei's average gene diversity for landraces was 0.377 and 0.305 for cultivars (Table 2). A lower mean average number of alleles, mean observed heterozygosity, mean Nei's average gene diversity and percentage of polymorphic loci at the 5 % level was observed in the cultivars compared to the landraces (Table 2), indicating that the landraces are genetically more diverse than the cultivars. Hence landraces are more line mixtures compared to cultivars. High average heterozygosity is observed both in the landraces (0.234) and cultivars (0.193). High heterozygosity means lots of genetic variability and might be an indication for the existence of high outcrossing in the landraces and cultivars. Heterozygosity enhances the level and stability of yielding performance in corn (Schnell and Becker 1986), sorghum (*Sorghum bicolor* L.) (Reich and Atkins 1970) and rapeseed (*Brassica napus*) (Léon 1991). In this study we observed high average heterozygosity both in landraces and cultivars. The existence of high hetrozygosity might enhance the level and stability of yielding performance in the sesame population. Great genetic diversity was observed in the Amhara and Oromia regions. This might be resulted from the broad environmental differences existing within these two regions. Landraces from these two regions might be used as source of materials for sesame breeding program.

3.3. Outcrossing rate estimates:

In this study a rough estimate of outcrossing from observed heterozygosity and Nei's average gene diversity was used. Different authors reported contradicting results concerning the outcrossing percentage of sesame (Ali and Alam,1933; Demetrios, 1980; Sun et al., 2015). We also observed a wide range of outcrossing in the landraces (9.1% to 96.4%) and cultivars (15.5% to 77.3%) with a mean of 45% and 46.4%, respectively (Table 2), indicating both the landraces and cultivars are not lines but line mixtures. Among the 47 germplasms evaluated, sixteen germplasms revealed out-crossing percentage more than 50%. The highest percentage of out-crossing was observed in landrace Acc11323 (t_e = 96.4) followed by Abunam (t_e = 83.3) and 7B (t_e = 78.7) , while landraces BG004 (t_e = 13.5), BCS010 (t_e = 11) and GA005 (t_e = 9.1) showed the least out-crossing percentage. The cultivars, Kelafo74 (t_e =77.3) and Mehado80 (t_e =15.5) showed the highest and lowest out-crossing percentage, respectively. In some cases the value of observed heterozygosity (H_o) was higher than the value of Nei's average gene diversity (H_e) resulting out-crossing values higher than 100% (Table 2) and those unreasonable results were omitted from the table. This may indicate the presence of heterozygote advantage or could be caused by the heterogeneity of environment in which the landraces are cultivated (Lowe et al., 2004). Another explanation might be isolate breaking effect (Wahlund, 1928). Out-crossing rate was highest in populations collected from Amhara region than those collected from other regions. The overall mean out-crossing rates in sesame were 48.23, 47.87, 45.02, 40.22, 36.5, 36.03, 31.5 for populations collected from Amhara, Oromia, Afar, Tigray, Benishangul-gumuz, Gambella and SNNP regions, respectively. The results suggest that outcrossing may vary among different regions with in the country. In studies of the natural outcrossing of sesame, different outcrossing percentage was reported by different authors (Ali and Alam, 1933; Demetrios, 1980; Sun et al., 2015) and in this study a wide range of outcrossing in the Ethiopian sesame population was observed. It can be concluded that sesame in general displays a very high level of outcrossing. The extremely high level of outcrossing detected in the present study indicates that increased vigour due to heterozygosity has been a major force in the course of evolution of Sesame.

Table 2 Summary of Genetic diversity measures of the 50 sesame population.

Population	Regions	Na[1]	H_o[2]	H_e[3]	P[4]	t_e[5](%)
Landraces						
BG019	Amhara	3.4	0.260	0.530	100	32.5
GA005	Gambella	2.8	0.080	0.480	100	9.1
EW011	Oromia	4.2	0.440	0.658	100	50.2
EW009	Oromia	3.0	0.380	0.488	90	63.8
WW001	Oromia	3.0	0.360	0.494	90	57.3
Shiraro	Tigray	2.4	0.220	0.400	80	37.9
Niguara	Tigray	2.8	0.220	0.488	90	29.1
Woldyakobo	Amhara	2.7	0.360	0.458	80	67.4

KeySelit	Amhara	3.2	0.340	0.586	80	40.9
BCS043	Afar	2.1	0.280	0.332	70	72.9
Acc11323	Amhara	2.1	0.320	0.326	60	96.4
BM52	Afar	2.9	0.300	0.528	100	39.7
Beshbesh	Tigray	1.6	0.320	0.244	50	*
Gobyo82	Amhara	2.3	0.300	0.360	60	71.4
Acc07	Tigray	1.4	0.260	0.154	30	*
BCS010	Afar	2.0	0.060	0.302	60	11
Tejin	Amhara	1.4	0.160	0.106	20	*
Acc212992	SNNPR	2.0	0.140	0.292	60	31.5
Dima	Tigray	1.8	0.140	0.222	40	46.1
Acc205	Amhara	2.2	0.140	0.362	70	24
Acc.08	Amhara	2.6	0.220	0.334	80	49.1
7B	Afar	2.4	0.280	0.318	80	78.7
Acc111502	Amhara	2.7	0.280	0.406	70	52.6
Population	Regions	Na^1	H_o^2	H_e^3	P^4	te^5
Landraces						
Abunam	Amhara	1.8	0.240	0.264	80	83.3
Acc202433	Amhara	2.8	0.420	0.484	100	76.6
J02	Oromia	3.1	0.380	0.498	90	61.7
Mikadra	Tigray	2.4	0.200	0.398	80	33.6
AccBG001	Benishangul - Gumuz	2.3	0.180	0.362	80	33.1
GA 009	Gambella	2.6	0.300	0.392	70	62
J01	Oromia	2.3	0.220	0.376	80	41.4
Tejahir	Amhara	1.7	0.160	0.212	40	60.6
Tajareb	Amhara	1.6	0.240	0.188	40	*
Hirhir	Tigray	2.2	0.200	0.392	80	34.2
J03	Oromia	2.6	0.220	0.426	90	34.8
G03	Gambella	2.4	0.200	0.370	80	37
Gojam Azene	Benishangul-Gumuz	2.1	0.240	0.318	80	60.6
B02	Oromia	1.8	0.120	0.206	60	41.1
BG004	Amhara	2.2	0.080	0.336	60	13.5
AccN5003	Amhara	2.7	0.200	0.410	80	32.3
Acc18	Amhara	3.3	0.220	0.518	90	27
Key Selit 2	Amhara	2.1	0.160	0.360	70	28.6
Hirhir 2	Amhara	2.3	0.100	0.372	80	15.5
BM50	Afar	2.4	0.200	0.408	90	32.5
Assosa	Benishangul-Gumuz	2.5	0.200	0.374	80	36.5
BCS022	Afar	2.6	0.240	0.460	80	35.3
Acc09	Tigray	2.0	0.220	0.292	60	60.4
Acc 208672	Oromia	2.9	0.220	0.446	80	32.7
Average		2.4	0.234	0.377	74.04	45
Cultivars						
Kelafo74		1.8	0.260	0.298	70	77.3
Mehado80		2.9	0.120	0.446	80	15.5
Tate		1.5	0.200	0.172	40	*
Average		2.0	0.193	0.305	63.33	46.4
Total mean		2.4	0.232	0.373	73.4	45.1

1 = Average number of alleles; 2= Observed heterozygosity;
3 = Nei's average gene diversity; 4 = Percentage of polymorphic loci at the 5 % level;
5 = The equilibrium outcrossing rate * t_e values omitted from the data

4. CONCLUSION

In crop improvement program development of uniform pure line cultivars has many advantages. Uniformity in maturity facilitates harvesting and uniformity in quality enhances the market value of the product. But pure lines are mainly effective under high input conditions. In this study high outcrossing have been estimated for landraces and cultivars, indicating both landraces and cultivars are not lines but line mixtures. Mixed lines may produce more stable yields over a greater range of environments and seasons, be more widely adapted, and offer broader protection against pests. In Ethiopia the areas where sesame production takes places have heterogeneous environment, line mixtures may be able to compensate for such heterozygous environmental conditions like the landraces do. Therefore, lines mixtures may be effective under low input condition and sever as a buffering mechanism for sesame production in Ethiopia. In conclusion, sesame cannot be clearly regarded as autogamous or allogamous, rather as a species with a mixed mating system.

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