J. ISSAAS Vol. 18, No. 2:45-54 (2012)

THE APPLICATION OF SSR MOLECULAR INDICATOR TO ASSESS THE PURITY AND GENETIC DIVERSITY OF WAXY CORN INBRED LINES

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(Received: January 22 2011; Accepted: July 20, 2012)

ABSTRACT

The analysis of 22 waxy corn inbred lines indicated that there were four lines having high homozygous rate: Waxy4, Waxy 9, Waxy16, Waxy 17 (under 7% of heterozygous rate) and 5 lines showed high heterozygous proportion (above 20%): Waxy 12, Waxy 14, Waxy18, Waxy 21, Waxy 22. The SSR primer system used in the experiments is relatively polymorphic with average value of PIC of 0.46. Classifying these lines into groups of heterosis by UPGMA method reveals that at genetic similarity coefficient of 0.38 the experimented lines were divided into six groups, including Group I: Waxy 10, group II: Waxy 16; Group III: Waxy 3, Waxy 5, Waxy 22, Waxy 21, Waxy 8, Waxy 9, Waxy 12, Waxy 18; Group IV: Waxy 2, Waxy 15, Waxy 20 , Waxy 17; Group V: Waxy 4, Waxy 6, Waxy 7, Waxy 11 and Group VI: Waxy 1, Waxy 14, and Waxy 19.

Key words: genetic distance, genetic similarity coefficient, heterosis, polymorphic, pedigree diagram

INTRODUCTION

Hybrid corn with high yield, high quality and interesting characteristics is dominating corn production all over the world. Single hybrid corn is created by the combination between two pure lines which have high combination ability. Therefore, the assessment of the purity and the combination in corn lines plays an important role in breeding hybrid corn. Traditional methods are used to evaluate the combination ability such as: top-cross, reciprocal cross that are based on the observation of morphological characteristics, require so much time and labor.

To quickly shorten the process of evaluation of the purity and the combination ability instead of using traditional methods, DNA molecular indicator is applied in molecular breeding to help breeders achieve many successes. Many techniques have been developed in molecular breeding such as: restriction fragment length polymorphisms (RFLP) (Botstein *et al.*, 1980), random amplified polymorphic DNAs (RAPD) (Williams *et al.*, 1990), amplified fragment length polymorphisms (AFLP) (Vos et al., 1995) and simple sequence repeats (SSR, microsatellites) (Tautz, 1989).

In a comparison of RFLP and SSR techniques, Smith and co-workers. (1997) stated that SSR markers offer advantages of reliability, reproducibility, discrimination, standardization, and cost effectiveness over RFLP and that SSRs represent the optimum approach for the identification and pedigree validation of maize genotypes. Using RFLP, RAPD, SSR and AFLP techniques on maize Pejic et al. (1998) supposed that both the SSR and AFLP technologies can replace RFLP. Garcia et

al. (2004) suggested AFLP markers are the best choice evaluation of diversity and assessing the relationships between tropical maize inbred lines.

The study sought to evaluate purity, genetic diversity of 22 waxy corn inbred lines and to classify them into groups by applying SSR molecular indicator.

MATERIALS AND METHODS

Twenty two waxy corn inbred lines that was collected by research group of Hanoi University of Agriculture:

No.	Name of line	Abbreviation	Code	Generations of breeding
1	Waxy 1	W1	CLT-N27	10
2	Waxy 2	W2	CLT-N8	10
3	Waxy 3	W3	CLT-N1	10
4	Waxy 4	W4	CLT-N6	10
5	Waxy 5	W5	CLT-N7	10
6	Waxy 6	W6	CLT-N11	10
7	Waxy 7	W7	CLT-N12	10
8	Waxy 8	W8	CLT-N2	10
9	Waxy 9	W9	CLT-N3	10
10	Waxy 10	W10	CLT-N4	10
11	Waxy 11	W11	CLT-N5	10
12	Waxy 12	W12	CLT-N10	10
13	Waxy 13	W13	CLT-N16	10
14	Waxy 14	W14	CLT-N18	10
15	Waxy 15	W15	CLT-N20	10
16	Waxy 16	W16	CLT-N22	10
17	Waxy 17	W17	CLT-N23	10
18	Waxy 18	W18	CLT-N24	10
19	Waxy 19	W19	CLT-N25	10
20	Waxy 20	W20	CLT-N26	10
21	Waxy 21	W21	CLT-N29	6
22	Waxy 22	W22	CLT-N14	10

DNA extraction method

CTAB (Cetyl Trimethyl Ammonium Bromide) protocol was used for DNA extraction (Saghai Maroof *et al.* 1984). Tissue powder was stirred into emulsion with a glass rod after adding 10 ml extraction buffer prewarmed to 65°C (1% CTAB, 0.7 M NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) and incubated at 65°C for one hour. Ten ml chloroform with isoamyl alcohol (24/1, *v/v)* was added and the mixture was shaken into the emulsion again. The mixture was centrifuged at the highest speed available (for example, 7000 rpm in *Hettich* Universal 16R) for 10 minutes at 25°C. Supernatant was mixed with 10µl RNase (10 mg/ml) in a fresh tube and laid on the bench for 10 minutes. DNA was precipitated with gentle shaking after adding 0.6 volume

isopropanol. DNA was hooked out and transferred into 70% ethanol for washing. After brief drying on Whatman 3mm paper, DNA was dissolved in TE (Tris-EDTA) buffer for use.

The concentration and purity of DNA was measured using a spectrophotometer (Ultrospec/Visible-65). The concentration of DNA was calculated using the following formula:

Concentration of DNA (µg/µl) = OD_{260} x 50 x 50 (µg/ml)/1000

With OD_{260} found at λ=260 nm

The purity of DNA was assessed by OD_{260}/ OD_{280} rate (OD_{280} is found index at λ=280 nm). If OD_{260}/ OD_{280} rate is from 1.8-2.0, we can conclude that DNA is pure.

PCR amplification and electrophoresis on acrylamide was conducted using the AMBIONET'process, 2004: PCR reaction in 25 µl contained 10 mM Tris-HCl (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM dNTPs, 60 ng/µl primer, 1 unit Taq DNA Polymerase (Life Technologies, USA) and 75-100 ng Template DNA. Amplification reaction consisted of preheating for 5 min at 94°C and of 35 cycles of 1 min at 94°C (denaturation) 1 min at 55°/ 61°C (annealing) and 3 min at 72°C (elongation) followed by 7 min at 72°C in Mastercycler Gradient PCR system (Eppendorf) . Amplified products were separated in 4% Typing Grade agarose gel (Life Technologies, USA), containing 0.5 ng/ml EtBr (Ethidium Bromide). Separated PCR products were visualized under UV light and photographed using Kodak Electrophoresis Documentation and Analysis System. Amplified products, which were less than 500 bp and where the polymorphism was difficult to detect in agarose gels were separated on denaturing long 6% polyacrylamide gels with a gel thickness of 0.4 mm. Gels were pre-run, samples denatured in loading buffer and electrophoresed for about 2 h or until the bromophenol blue remained visible. The molecular weight markers used were 25 bp ladder (Life Technologies). Gels were stained with silver nitrate, according to the protocol by Promega. After staining, gels were air dried and photographed.

Molecular data was collected by scoring 1 for present of band, 0 for absence of band and 9 for missing data. The collected data was analyzed using NTSYS-pc 2.1.

Targets: Coefficient of PIC (Polymorphic Information Content)

PIC = 1-ΣPi, where Pi is the frequency of occurrence of i^{th} allele

The heterozygous rate (H%) of per corn line was calculated as:

$$H\% = \frac{\text{Number of SSR primers appeared} \geq \text{2allele/1locus}}{\text{Number of amplified primers}} \times 100$$

The percentage of missing data per line for corn (M% line) and for each primer (M% primer) was calculated as:

$$M\%\ \text{line} = \frac{\text{Number of primers missing data}}{\text{Total of primers}} \times 100$$

$$M\%\ \text{primer} = \frac{\text{Number of lines missing data}}{\text{Total of research lines}} \times 100$$

The genetic distance was calculated using the formula: D = 1 - I

where D is the genetic distance, I is Jaccard index or Jaccard similarity coefficient (Lanza *et al.* 1997)

Corn lines were classified into groups of heterosis using the UPGMA (Unweighted Pair Group Method with Arithmetic Average) method (George *et al.* 2004).

RESULTS AND DISCUSSION

Genetic purity

The 22 lines having average of missing data was 6.95% (Table 1) fluctuating from 0.00 to 11.43%, lower than the threshold (15%).

The average heterozygous rate was 16.07% and ranged from 6.06 to 48.48%. In which there were four lines having high genetic purity as 4 Waxy, Waxy 9, Waxy 16, Waxy 17 (under 7% of heterozygous rate). Results also showed that 5 lines had higher heterozygous rate than 20% (threshold rate) including: Waxy12, Waxy 14, Waxy18, Waxy 21, and Waxy 22. For example, amplification of profiles of 22 waxy corn lines of locus phi053 showed that Waxy 14, Waxy18, Waxy 21 and Waxy 22 have two alleles at this locus (Fig.1). Thus, these lines having low genetic purity should be made more pure and should not be used for crossing.

Table 1. The percentage of missing data (%M) and the heterozygous rate (%H) of 22 waxy maize inbred lines based on 35 SSR loci.

No.	Name of corn line	Percentage of missing data (%)	Heterozygous rate(%)
1	Waxy 1	11.43	16.13
2	Waxy 2	5.71	9.09
3	Waxy 3	5.71	9.09
4	Waxy 4	5.71	6.06
5	Waxy 5	5.71	18.18
6	Waxy 6	8.57	12.50
7	Waxy 7	5.71	9.09
8	Waxy 8	10.14	17.24
9	Waxy 9	8.57	6.25
10	Waxy 10	8.57	18.75
11	Waxy 11	8.57	12.50
12	Waxy 12	11.43	22.58
13	Waxy 13	8.57	15.63
14	Waxy 14	0.00	25.71
15	Waxy 15	5.71	9.09
16	Waxy 16	5.71	6.06
17	Waxy 17	8.57	6.25
18	Waxy 18	5.71	48.48
19	Waxy 19	5.71	12.12
20	Waxy 20	5.71	9.09
21	Waxy 21	5.71	36.36
22	Waxy 22	5.71	27.27
	Average	6.95	16.07

Fig. 1. Amplification profiles in 22 waxy corn inbred lines (from 1 to 22) using phi053 primer (M: ØX174 DNA/HinfI marker)

Polymorphism Information Content (PIC)

Polymorphism Information Content (PIC) is a measure of diversity allele at a locus. Results presented in Table 2 shows the value varied from the lowest 0.13 (phi102228) to the highest 0.72 (umc1136) and average 0.46. The number of allele per locus ranged from 1 to 6 and had an average was 3.26. This result showed that the SSR primers used in the analysis is relatively polymorphic. However, phi102228 and phi089 primers having low PIC value (0.13 and 0.18, respectively) should not be used to genetic diversity analysis of the other waxy corn lines. Beside that, primers umc1277, phi328175 and phi227562 have the rate of missing data higher than 20% (threshold rate). The data of these primers were removed before doing tree analysis**.**

Table 2. Numbers of allele, PIC value and percentage of missing data of 35 SSR primers

No.	Name of primer	Position on chromosome	Type of repeat	Number of allele	Size (bp)	Percentage of missing data (%)	PIC value
1	phi089	6.08	ATGC	2	87-95	9.09	0.18
2	phi053	3.05	ATAC	6	169-195	4.55	0.62
3	phi029	3.04	AG/AGCG***	6	148-162	4.55	0.65
4	phi087	5.06	ACC	4	150-177	13.64	0.23
5	phi374118	3.02	ACC	2	217-238	13.64	0.43
6	phi127	2.08	AGAC	3	112-126	9.09	0.33
7	nc130	5.00	AGC	3	140-148	9.09	0.46
8	phi109642	2.00	ACGG	2	136-144	4.55	0.34
9	umc1277	9.08	(AATA)5	2	134-138	27.27	0.43
10	umc1279	9.00	(CCT) 6	3	92-101	9.09	0.50
11	phi 072	4.00	AAAC	3	143-167	18.18	0.57

No.	Name of primer	Position on chromosome	Type of repeat	Number of allele	Size (bp)	Percentage of missing data (%)	PIC value
12	phi 083	2.04	AGCT	4	125-137	4.55	0.66
13	umc1304	8.02	(TCGA) 4	2	129-137	0.00	0.24
14	phi452693	6.06	AGCC	6	125-145	4.55	0.70
15	phi448880	9.05	AAG	4	173-188	0.00	0.67
16	umc1066	7.01	(GCCAGA)5	4	139–158	0.00	0.56
17	phi108411	9.06	AGCT	2	125-129	9.09	0.50
18	phi423796	6.01	AGATG	4	121-141	0.00	0.46
19	phi328175	7.04	AGG	3	100-130	22.73	0.66
20	phi102228	3.04-.05	AAGC	2	123-131	0.00	0.13
21	phi109275	1.00	AGCT	4	117-143	0.00	0.64
22	phi065	9.03	CACTT	3	131-151	0.00	0.16
23	phi032	9.04	AAAG	3	233-241	4.55	0.45
24	phi299852	6.08	AGC	4	96-151	18.18	0.48
25	phi109188	5.00	AAAG	4	148-174	13.64	0.52
26	umc1153	5.09	(TCA)4	3	105-114	9.09	0.27
27	umc1136	3.10	GCA	5	132-159	0.00	0.72
28	phi227562	1.12	ACC	3	307-328	22.73	0.64
29	umc1109	4.10	ACG	3	104-116	0.00	0.24
30	umc1196	10.07	CACACG	4	137-161	0.00	0.32
31	phi100175	8.06	AAGC	2	117-141	13.64	0.48
32	phi 076	4.11	AGCGGG	3	161-173	4.55	0.57
33	phi233376	8.03	CCG	4	142-154	0.00	0.47
34	umc1061	10.06	(TCG)6	3	101-110	4.55	0.44
35	phi339017	1.03	AGG	2	148-163	0.00	0.43
	Average			3.26		7.27	0.46

Genetic diversity and pedigree diagram

The experimental results showed that genetic similar coefficient of lines ranged from 0.23 to 0.71. In general, genetic distance of research lines was far. Thus, these lines had different from genetic material. This is one of big opportunities for selecting hybrid waxy corn from inbred lines.

Classifying lines into groups of heterosis by UPGMA method reveals that at genetic similarity coefficient of 0.38 the experimented lines are divided into six groups:

Group I: Waxy 10
Group II: Waxy 16
Group III: Waxy 3, Waxy 5, Waxy 22, Waxy 21, Waxy 8, Waxy 9, Waxy 12, Waxy 18
Group IV: Waxy 2, Waxy 15, Waxy 20, Waxy 17
Group V: Waxy 4, Waxy 6, Waxy 7, Waxy 11, Waxy 13
Group VI: Waxy 1, Waxy 14, Waxy 19

Some researches indicated that the correlation between the genetic distance of parents and the heterosis of their hybrids was positive (Drinic et al 2002; Aguiar et al. 2008). In this research, most of lines had far genetic distance (except waxy 6 and waxy 7). This result leads to a potential in selecting new hybrid corn with high heterosis. Waxy 10 and waxy 16 may be used to test with other lines in this colony.

The grouping of lines, based on genetic distance as determined by the molecular markers, will be an important base in the use, exploitation of materials as well as selection of new varieties. The probability of creating hybrids having high heterosis from crossing among different groups will be higher than those crossed in the same group.

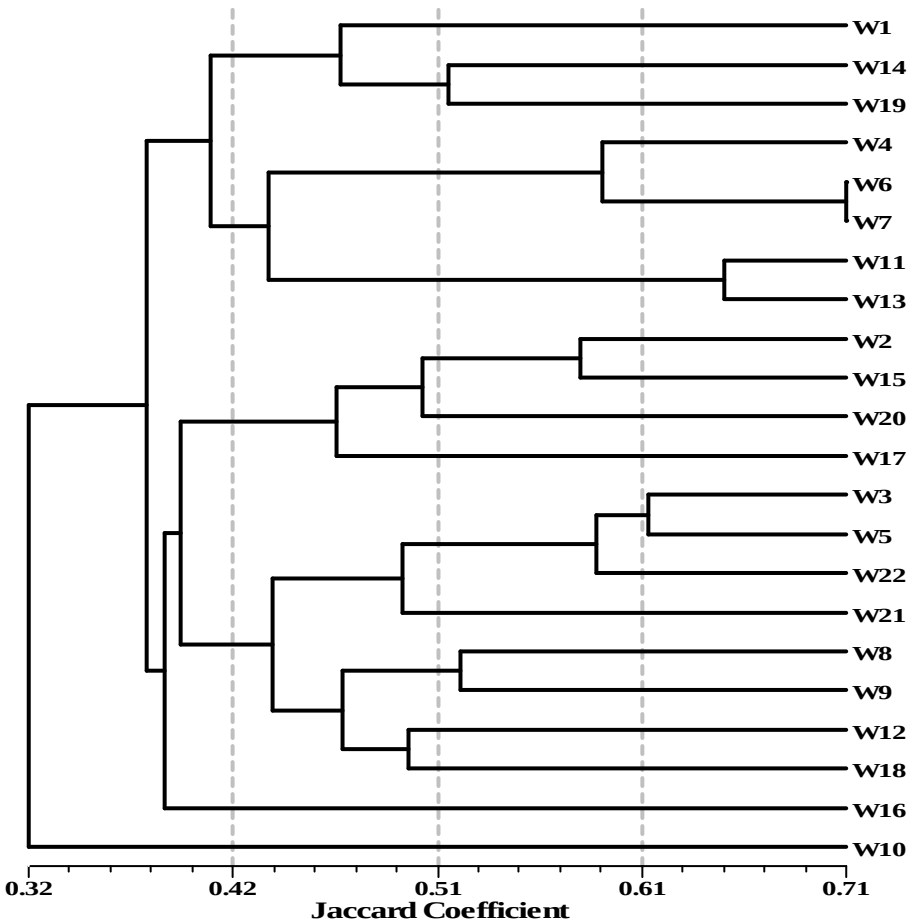

Fig. 2. Pedigree diagram of 22 Waxy (W) corn inbred lines by UPGMA grouping method

Table 3. Genetic similarity coefficient of 22 waxy corn inbred lines

Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Waxy 1	1.00																					
Waxy 2	0.52	1.00																				
Waxy 3	0.49	0.53	1.00																			
Waxy 4	0.45	0.40	0.41	1.00																		
Waxy 5	0.47	0.54	0.61	0.49	1.00																	
Waxy 6	0.42	0.44	0.41	0.65	0.53	1.00																
Waxy 7	0.52	0.44	0.39	0.53	0.44	0.71	1.00															
Waxy 8	0.35	0.47	0.49	0.43	0.48	0.49	0.43	1.00														
Waxy 9	0.35	0.39	0.33	0.38	0.43	0.50	0.52	0.52	1.00													
Waxy 10	0.33	0.33	0.28	0.29	0.39	0.29	0.31	0.31	0.29	1.00												
Waxy 11	0.39	0.31	0.29	0.51	0.38	0.42	0.40	0.30	0.32	0.26	1.00											
Waxy 12	0.30	0.29	0.29	0.35	0.56	0.36	0.32	0.52	0.42	0.29	0.29	1.00										
Waxy 13	0.44	0.34	0.38	0.48	0.43	0.38	0.42	0.36	0.31	0.31	0.65	0.33	1.00									
Waxy 14	0.50	0.43	0.40	0.40	0.40	0.36	0.42	0.41	0.40	0.32	0.32	0.33	0.32	1.00								
Waxy 15	0.40	0.58	0.42	0.34	0.46	0.37	0.30	0.42	0.23	0.37	0.37	0.30	0.33	0.30	1.00							
Waxy 16	0.37	0.38	0.40	0.35	0.49	0.43	0.36	0.40	0.26	0.28	0.32	0.37	0.31	0.35	0.38	1.00						
Waxy 17	0.37	0.53	0.37	0.35	0.49	0.43	0.35	0.49	0.33	0.27	0.33	0.33	0.38	0.35	0.45	0.38	1.00					
Waxy 18	0.39	0.36	0.41	0.36	0.46	0.40	0.42	0.48	0.45	0.41	0.32	0.50	0.42	0.40	0.31	0.34	0.46	1.00				
Waxy 19	0.44	0.36	0.36	0.41	0.36	0.34	0.37	0.35	0.25	0.30	0.50	0.33	0.42	0.52	0.43	0.46	0.27	0.27	1.00			
Waxy 20	0.38	0.46	0.37	0.31	0.37	0.37	0.32	0.42	0.27	0.29	0.28	0.29	0.35	0.25	0.56	0.38	0.39	0.33	0.35	1.00		
Waxy 21	0.33	0.43	0.43	0.38	0.53	0.38	0.37	0.47	0.45	0.39	0.29	0.39	0.33	0.39	0.36	0.44	0.38	0.46	0.36	0.39	1.00	
Waxy 22	0.41	0.45	0.58	0.47	0.60	0.48	0.45	0.52	0.41	0.43	0.37	0.44	0.43	0.50	0.35	0.40	0.37	0.40	0.42	0.42	0.54	1.00

CONCLUSION

The analysis of 22 waxy corn inbred lines indicates that there were four lines having high homozygous rate: Waxy 4, Waxy 9, Waxy16, Waxy 17 (lower than 7% of heterozygous rate) and 5 lines showed high heterozygous proportion (higher than 20%): Waxy 12, Waxy 14, Waxy18, Waxy 21, and Waxy 22.

The SSR primer system used in the experiments was relatively polymorphic with a PIC average value of 0.46.

Classifying these waxy corn inbred lines into groups of heterosis by UPGMA method reveals that at genetic similarity coefficient of 0.38 the experimented lines were divided into six groups, including Group I: Waxy 10, Group II: Waxy 16; Group III: Waxy 3, Waxy 5, Waxy 22, Waxy 21, Waxy 8, Waxy 9, Waxy 12, Waxy 18; Group IV: Waxy 2, Waxy 15, Waxy 20, Waxy 17; Group V: Waxy 4, Waxy 6, Waxy 7, Waxy 11 and Group VI: Waxy 1, Waxy 14, Waxy 19.

ACKNOWLEDGEMENT

This work was supported by the Official Development Assistance of the World Bank.

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