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CHARACTERIZATION OF A SET OF SSR MARKERS FOR MAIZE GENOTYPING AND ESTIMATION OF SSR ANALYSIS COST FOR ROUTINE USE

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CHARACTERIZATION OF A SET OF SSR MARKERS FOR MAIZE GENOTYPING AND ESTIMATION OF SSR ANALYSIS COST FOR ROUTINE USE.

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Abstract

Our presentation will describe an efficient SSR analysis system using a LI-COR automated DNA analysis system. This system consists of (1) multiplexing PCR, (2) tailed one of the two primers for each SSR primer pair, (3) pooling two independent PCR runs each using a different dye for tail labelling before gel loading, and (4) reloading at least three times a same gel. Secondly, a set of 50 SSR markers, well distributed on the maize genome, was established for routine genotyping study in maize, after their characterization on a set of 45 public maize inbred lines. The cost of routine genotyping using our SSR analysis condition will be estimated from sample preparation, DNA extraction, PCR and to data analysis.

Introduction

Many molecular marker systems are available in maize. A lot of molecular data have been generated, including those using SSR markers (Taramino and Tingey, 1996; Smith et al. 1997; Senior et al. 1998). The present study aimed (1) to establish a semi-automated SSR analysis conditions, (2) to select and characterize a set of SSR markers suitable for genotyping, and (3) to estimate the cost of routine analyse.

Materials and methods

SSR primer pairs used: A set of 60 SSR primer pairs was proposed by the team of Pr A. Melchinger, University of Hohenhein, Germany, in the context of the EU research program "GEDILUX" (contract n° QLRT-2000-00934). All those SSR markers are publicly available: the primer sequences and mapping information can be found via http:/www.agron.missouri.edu/body/ssr.html.

SSR analysis conditions: SSR analysis has been realized, using a LI-COR automated DNA analysis system. The PCR reactions were performed in 10 µl containing 1x PCR buffer, 0.125 mM each of dNTPs, 3 mM $MgCl_2$, 0.25 µM each primer, 0.5 U AmpliTaq Gold (Applied Biosystems) and 10 ng template DNA. Primers were labelled by fluorescence dyes 700 or 800, using a tailed primer strategy. A 'touchdown' program was used for PCR amplification; it consists of an initial denaturation step at 95°C for 10 min, followed by 10 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 30 s, with decreasing 1 °C per cycle of the annealing temperature from 64°C to a touchdown at 55°C, and 30 cycles of 94°C for 30 s, 55°C for 30 s

and 72°C for 30 s, finished at 72°C for 10 min. PCR products were run on a 5% denaturing acryl amide gel.

Plant materials: Forty-five public inbred lines were used for the characterization of the SSRs selected. 20 seeds were pooled and grounded together. About 100 mg of the flour were submitted for DNA isolation, using the DNeasy Plant Mini kit (QIAGEN).

Results and discussion

Establishment of SSR analysis conditions using a LI-COR automated DNA analysis system

The described reaction conditions work very well. To avoid appearance of spurious amplifications, a "touchdown" PCR program (Don et al. 1991) has been performed. Moreover, "touchdown" strategy is essential to use a same PCR program for the amplifications of a maximum of SSR primers having different melting temperatures. This was our case; the great majority of the SSRs functioned well under the "touchdown" PCR program described. Only those SSRs were selected among the 60 SSRs initially provided by university of Hohenheim.

To optimize the cost of SSR analysis for routine analysis using a LI-COR DNA analysis system, we performed (1) a tailed primer technique (Fig. 1), and (2) multiplexing PCR and gel electrophoresis (Fig. 2). Two tails were used: M13 (5'-CACGACGTTGTAAAACGAC-3') and 35S1 (5'-GCTCCTACAAATGCCATCA-3'). The first tail is an universal primer and the second one is derived from Cauliflower Mosaic Virus 35S promoter (Pietsch et al. 1997). Usually one of the two tails is added to the 5'-end of one of the SSR primer pair (forward or reverse) during the primer synthesis. Three primers are necessary for the amplification of each SSR locus: one tailed forward primer, one normal reverse primer and one labelled tail. Using this strategy, we needed only to label two tails for amplifying all the SSRs used in this study. This has led to a significant savings of money. Two tails are needed if the PCR products of two independent PCR runs, one using the dye 700 and the other using the dye 800 for tail labelling, are pooled before gel loading. All the two tails worked well in our conditions for all SSRs used, even if the tail 35S1 gives generally better amplification than that of the tail M13. For multiplexing PCR, several SSR primer pairs were combined together according to allele sizes. The competition between primer pairs was the main obstacle for establishing the functional PCR multiplexes. It should indicate that in case of competition among primers it's always the SSR amplifying the largest alleles in size which failed to amplify. Compatible primers sets can be obtained by empirical experiences. Two to five SSR markers can be multiplexed together. In order to avoid irregular amplifications, only 2-3 primer pairs are multiplexed in routine analysis. On the contrary, there was no particular difficulty to perform multiplexing electrophoresis. One same gel can be easily reloaded at least 3 times. It should be indicated that high quality of DNA solutions is required for multiplex PCR. That's why we used the Plant DNeasy kit.

Selection and characterization of a set of SSR markers for genotyping maize inbred lines

Polymorphism level: Out of the 60 SSRs initially provided by university of Hohenteim, 51 have been finally chosen for our study. These SSRs function well in our conditions of analysis. Table 1 shows the general information on the 51 SSR markers: genome coverage by chromosome, number of alleles generated across the 45 maize inbred lines used, range of allele size and PIC (Polymorphism Information Content) value. There are on average 5 SSR markers per chromosome, with a min of 3 and a max of 7. The number of alleles per SSR

locus ranged from 2 to 7, with an average of 3.8. The value of PIC is varied from 0.16 to 0.80, with a mean of 0.57. So these SSR markers have a good polymorphism level, except a few of SSRs.

Level of fixation of SSR loci and inbred lines: A total of 2295 data points (51 SSRs x 45 lines) were generated. Among them, 139 cases show heterogeneity (601%). This level of heterogeneity is more or less high for maize inbred lines. The great majority of the 45 lines analysed are old inbreds which are no more used for modern breeding. Among the inbreds, only 14 lines (31.1%) do not show heterogeneity. The others show heterogeneity at one to 21 SSRs loci. At level of SSRs, only 5 SSR loci are well fixed across all the inbred lines. Six SSRs detected heterogeneity at least on 5 maize inbred lines; they are phi114, phi065, phi083, umc1329, umc1641 and phi089. The last two SSRs detected heterogeneity on 10 and 13 maize inbred lines respectively.

Multiplexing PCR: Twenty-three functional multiplex primers combinations have been established by the present work (Table 2), including 15 triplex and 8 duplex combinations. However, we have not succeeded in multiplexing 3 SSR markers. Certain SSRs work in two or more multiplex PCR combinations.

Estimation of SSR cost for routine analysis

In our conditions of SSR analysis, we can achieve at:

- grounding 20 samples of 20 seeds per hour and per technician, ⇨ **3 minutes** per sample
- performing 196 extractions per 7.5 hours and per technician, ⇨ **3 minutes** per sample
- carrying out PCR + gel electrophoresis for 96 samples x 3 SSRs in 40 minutes by a technician, ⇨ **0.14 minutes** per data point
- analysing gel and data for 96 samples x 3 SSRs in 60 minutes by a technician, ⇨ **0.21 minutes** per data point

The hourly rate for a technician is about 17 € in GEVES. We multiply by a factor of two, by taking into account all overhead costs (amortization of equipments and premises, maintenance of equipments, formation and holidays of personnel, etc…). So we will use an hourly rate of 34€ for a technician for all our cost estimation.

The whole cost of SSR analysis can be divided en two components:

- 1) sample preparation and DNA extraction which cost per sample:
  - comsummables: 1€
  - personnel cost: 6 min X 34 €/60 = 3.4€
  - **Total: 4.4 €.**

- 2) data production and analysis which cost per data point:
  - comsummables: 0.1€
  - personnel cost: (0.14 min +0.21 min) X 34 €/60 = 0.2€
  - **Total: 0.3 €.**

The cost for producing one data point in our conditions will be varied according to the number of SSR markers used for a whole analysis:

- using only 1 SSR: 4.4€ + 0.3€ = 4.7€
- using 5 SSRs: 4.4€/5 + 0.3€ = 1.18€
- using 10 SSRs: 4.4€/10 + 0.3€ = 0.74€
- using 30 SSRs: 4.4€/30 + 0.3€ = 0.45€
- using 50 SSRs: 4.4€/50 + 0.3€ = 0.39€

So it's evident that the more SSR markers are used and lesser the cost of data point is for SSR analysis. When more than five SSR markers are used for an analysis, the cost per data point will drop around one Euro. This cost becomes interesting and supportable for routine analysis. In the case of 30 SSR markers used, the data point cost will be only 0.39 Euro.

The present study permitted the establishment of a very efficient SSR analysis conditions in maize, using a LI-COR automated DNA analysis system. This system works also very well in our laboratory in sunflower, oilseed rape and wheat. A set of 51 SSR loci were selected and characterized across 45 maize inbred lines. They are functional in our conditions with system. All these SSR markers are single simple co-dominant markers. These SSR markers can be used for variety identification. According to the type of testing performed (variety description, purity testing, hybrid formula verification, variety identity checking in the process of seed certification, etc…), one can chose more or less SSRs to be used. The cost of SSR analysis, based on the real experience and practice in our laboratory, has been estimated. This cost can be extrapolated to other species, using the same SSR analysis system and conditions.

Acknowledgements
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References

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research 19:4008.

Pietsch K, Waiblinger U, Brodmann P, Wurz A (1997) Scrreningverfahren zur identifizierung gentechnisch veraenderter lebensmittel. Deutsche Lebensmittel RundschauHeft 2: 35-38.

Senior ML, Murphy JP, Goodman MM, Stuber CW (1998) Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. Crop Sci. 38: 1088-1098.

Smith JSC, Chin ECL, Shu H, Smith OS, Wall SJ, Senior ML, Mitchell SE, Kresovich S, Ziegle J (1997) An evaluation of the utility of SSR loci as molecular markers in maize (Zea mays L.) : comparisons with data from RFLPS and pedigree. Theor Appl Genet 95: 163-173.

Tang S, Knapp SJ (2003) Microsatellites uncover extraordinary molecular genetic diversity in native American land races and wild population of cultivated sunflower. Theor Appl Genet (in press).

Taramino G, Tingey S (1996) Simple sequence repeats for germplasm analysis and mapping in maize. Genome 39: 277-287.

Table 1 Number of alleles and the value of PIC of the 51 SSRs obtained by genotyping the 45 maize inbred lines

Chromosome	SSR	number of alleles	Range of allele size (bp)	PIC value
1	**phi 109275**	5	123-138	0.66
	phi 427913	4	123-135	0.52
	phi 011	4	213-228	0.65
	phi 064	7	84-108	0.80
2	**phi 083**	4	123-135	0.65
	phi 127	4	111-126	0.68
	phi 101049	6	219-270	0.75
3	**phi 104127**	2	153-162	0.49
	phi 102228	3	123-129	0.58
	phi 053	5	168-210	0.65
	umc 1489	3	126-135	0.45
	umc 1641	6	192-219	0.68
4	**phi 072**	3	141-162	0.58
	phi 213984	2	285-303	0.16
	phi 308090	2	219-222	0.44
	phi 079	3	177-192	0.61
	umc 1329	3	78-93	0.64
	phi 093	4	282-294	0.65
	umc 1180	2	102-105	0.50
5	**nc 130**	2	141-144	0.48
	phi 396160	3	288-309	0.42
	phi 331888	4	126-135	0.45
	phi 333597	3	210-216	0.57
	phi 128	3	99-111	0.60
	umc 1153	4	102-114	0.66
6	**umc 1143**	5	72-87	0.73
	phi 423796	5	123-138	0.41
	umc 1887	4	90-99	0.67
	phi 452693	5	123-141	0.36
7	**phi 123**	3	141-147	0.51
	phi 089	3	84-93	0.46
	umc 1545	4	69-81	0.62
	phi 114	5	135-168	0.69
	phi 069	4	189-204	0.67
	phi 116	4	150-168	0.72
8	**phi 420701**	4	288-300	0.75
	umc 1304	2	123-135	0.48
	phi 233376	5	138-159	0.67
	umc 1161	5	132-150	0.74
	phi 100175	3	132-141	0.67
	phi 015	4	81-102	0.57
9	**umc 1279**	3	93-102	0.28
	phi 065	4	129-156	0.63
	phi 032	3	231-240	0.48
	phi 448880	3	171-186	0.52
	umc 1675	4	153-162	0.58
10	**phi 041**	4	195-213	0.74
	umc 1152	6	153-174	0.57
	phi 050	3	81-87	0.37
	phi 084	2	156-159	0.50
	umc 1061	4	99-108	0.54
Mean		**3.8**		**0.57**

Table 2 Functional combinations of SSRs on multiplexing PCR in maize, some of SSRs function in several combinations

Combination	Multiplex	SSR markers
1	Triplex	phi015/phi109275/phi053
2	Triplex	umc1143/phi423796/phi448880
3	Triplex	phi333597/phi448880/umc1161
4	Triplex	phi333597/phi448880/phi233376
5	Triplex	phi333597/phi452693/umc1152
6	Triplex	umc1489/umc1180/phi084
7	Triplex	phi308090/umc1122/umc1153
8	Triplex	phi374118/phi079/phi127
9	Triplex	phi079/phi128/umc072
10	Triplex	phi069/phi116/umc1887
11	Triplex	phi96100/phi084/phi083
12	Triplex	phi213984/phi032/phi079
13	Triplex	umc1641/nc130/phi064
14	Triplex	phi101049/phi104127/phi331888
15	Triplex	phi089/phi123/phi396160
16	Duplex	phi427913/umc1061
17	Duplex	phi114/umc1061
18	Duplex	umc1675/phi050
19	Duplex	phi093/phi011
20	Duplex	umc1545/umc1304
21	Duplex	phi065/umc1279
22	Duplex	phi065/phi420701
23	Duplex	phi089/phi100175
24	Simplex	phi 041
25	Simplex	phi 102228
26	Simplex	umc 1169

Fig.1. Tailed primer strategy

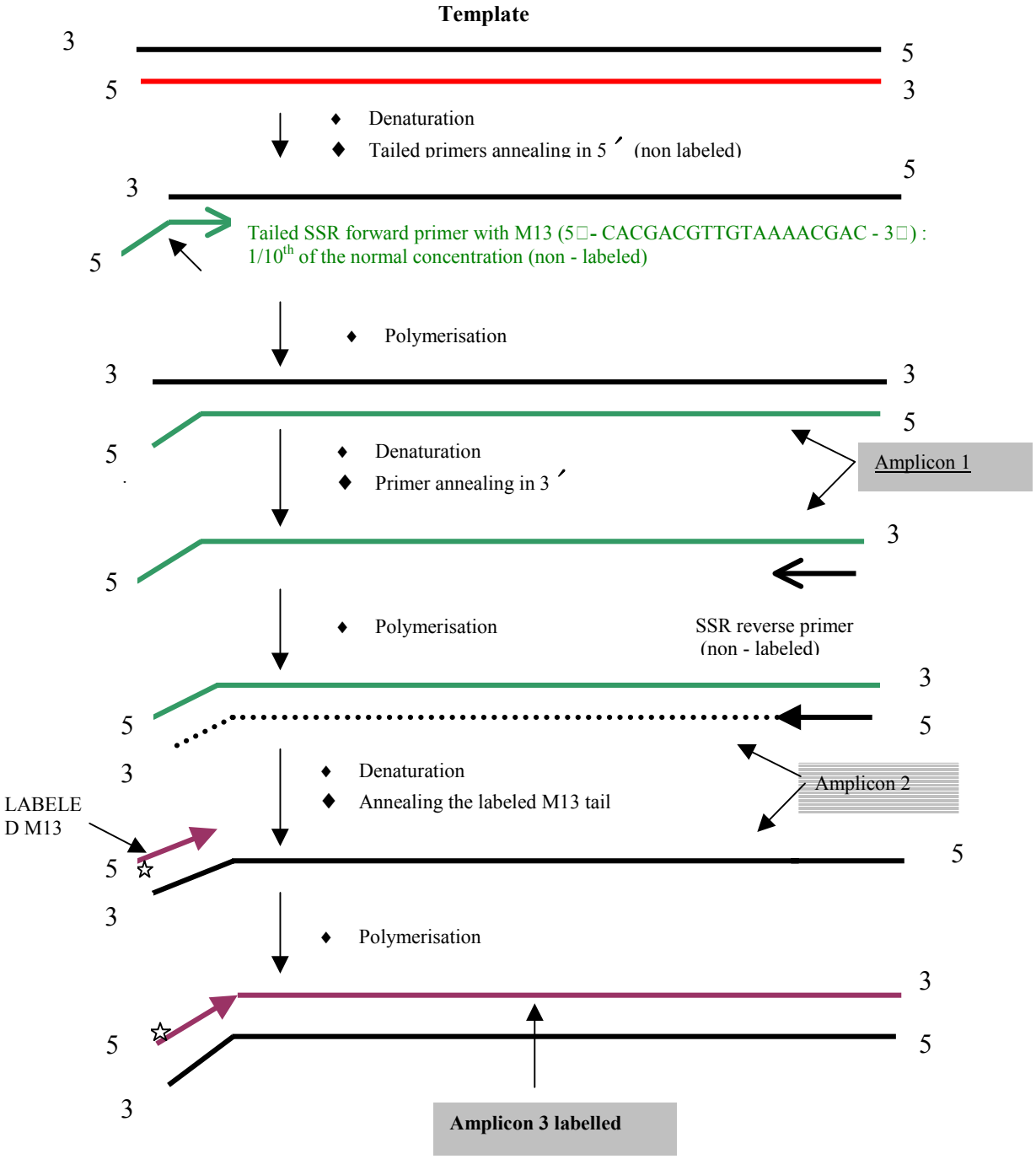
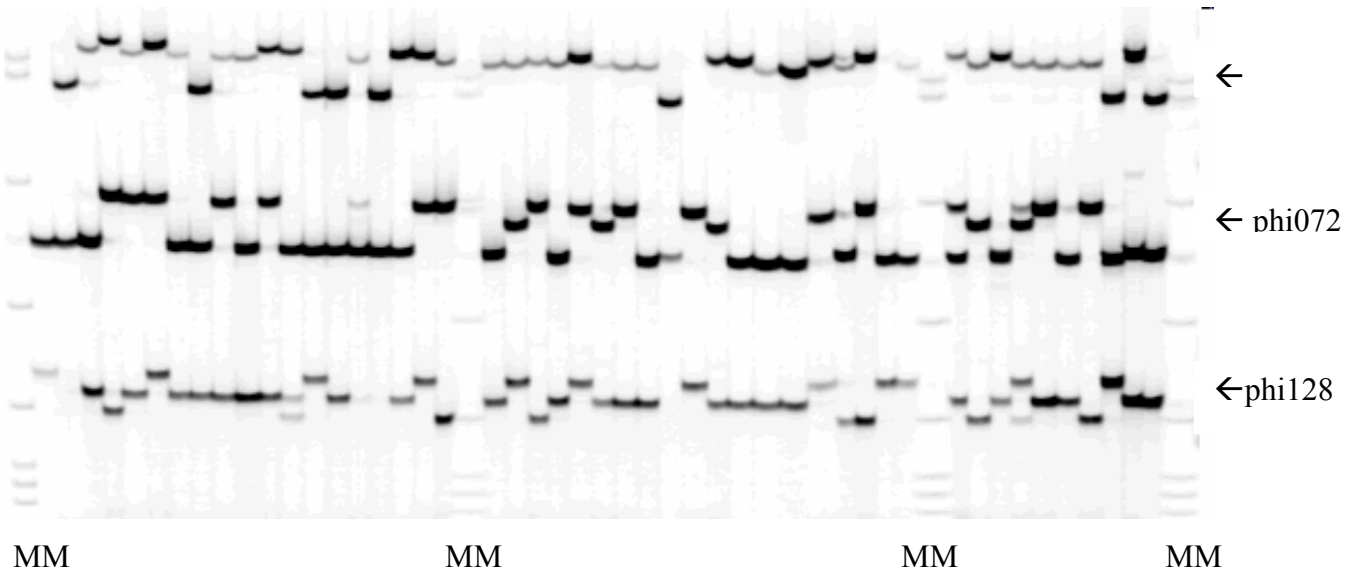


Figure 2. Image of a triplex SSR gel on the 45 maize inbred lines. MM = molecular weight markers.



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