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Full Length Research Paper

Optimization of microsatellite DNA Gelred fluorescence imaging technology

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Gelred fluorescent dye has broader prospects of application in DNA experiment because of its high sensitivity, security and stability. In order to explore the best microsatellite DNA Gelred imaging technology, this study compared its dosage by using three methods; precasting gels method (PG), staining sample method (SS) and immersion gels method (IG). The results show that agarose gel electrophoresis (AGE) fluorescence imaging technology can use the first method (PG) and the concentration of Gelred was 1X, because of the best banding and easy operation. The polyacrylamide gel electrophoresis (PAGE) can use the third method (IG), for the advantages of clear and bright image, saving dye and easily redying to image. The orthogonal test showed that the parameters of IG method were: the concentration of Gelred was 2X, that of sodium chloride was 10% and immersion time was 40 min. The optimization of microsatellite DNA Gelred fluorescence imaging technology would lay a technical foundation in DNA banding related experiments.

Key word: Agarose gel electrophoresis (AGE), polyacrylamide gel electrophoresis (PAGE), fluorescence imaging technology of Gelred, simple sequence repeat (SSR).

INTRODUCTION

At present, simple sequence repeat (SSR) has been a very widespread process in germplasm resources analysis and character identification breeding practice, because it is highly reproducible, polymorphic and generally codominant. The most current molecular markers detection methods are agarose gel electrophoresis (AGE) with ethidium bromide (EB) (Lunn et al., 1990; Seville et al., 2001), and polyacrylamide gel electrophoresis (PAGE) argentation (Liu et al., 1996; Pan et al., 2001; Zhebentyayeva, 2003). However, as a kind of strong mutagen, EB has high carcinogenicity (Chang et al., 2010; Morin and Smith, 1995; Kiltie et al., 1997; Zhao et al., 2007). In addition, argentation steps red tape, reagents of the fixer and developer solution also have different degree of toxicity. So, many researchers are working hard at exploring more safety and sensitive dying and staining methods. Lou et al. (2011) contrasted Gelred, GoldView and EB; the experimental result show that Gelred was the best on dying effect, next was EB. Liu et al. (2011) found a new DNA band display technology; used Gelred instead of argentation and mixed PCR products, and dying to electrophores directly, took photos and the bands were also clear. However, there were some short comings in the application of the new technology by other researchers, and the procedure should be optimized. So, the author analyzed the different usage methods and dosage of Gelred in AGE and PAGE to perfect the technology and lay a technical foundation in DNA banding related experiments.

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MATERIALS AND METHODS

Different varieties of *Armeniaca cathayana* D. L. (Fu et al., 2010) were collected from Zhangjiakou, Hebei province. One pair of the primer was from SSRs (BPPCT023); positive sequence: TGCAGCTCATTACCTTTTGC, reverse sequence: AGATGTGCTCGTAGTTCGGAC. Another was from GeneBank (named 7); positive sequence: AGCTCGCAAACCCTGTAAAA, reverse sequence: GCTGGTCTGAGTTCGAGGAC. Reagent

Table 1. Three factors and three levels in method IG.

Factor level	Concentration of Gelred (X)	Concentration of NaCl (%)	Immersion time (min)
1	1	5	20
2	2	10	30
3	3	15	40

Table 2. Orthogonal design and dyeing effects of IG [$L_9(3^4)$].

Dispose of combination	Concentration of Gelred (X)	Concentration of NaCl (%)	Immersion time (min)	Banding effect (ranking)
1	1	5	20	1
2	1	10	30	2
3	1	15	40	3
4	2	5	30	3
5	2	10	40	5
6	2	15	20	1
7	3	5	40	4
8	3	10	20	2
9	3	15	30	3

(Gelred, marker etc.) was bought from Takara company.

DNA isolation and PCR amplification

Total genomic DNA was extracted by the CTAB procedure. The DNA concentration was measured using a spectrophotometer and checked on 1% TAE agarose gels. Each PCR reaction (20 µl final volume) contained 9 µl of Premix Taq Version 2.0 (Loading dye mix), 1 µl of each primer (10µmoL/L), 1.5 µl of DNA (20mg/L) and 7.5 µl of ddH_2O. The cycling parameters were: 4 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 53°C and 80 s at 72°C, with a final step of 10 min at 72°C.

Agarose gel electrophoresis (AGE)

Precasting gels method (PG)

Gelred 10000X stock reagent was diluted in the 1.5% agarose gel solution at 1:10000. Then, the gel was cast at room temperature to solidify. Next, 5 µl of PCR products were electrophoresed for 50 min at 150 V; finally, the gel was put in an ultraviolet analysis device immediately for photo analysis.

On the other hand, dyeing auxiliary NaCl was added into gel solution for a gradient experiment; the concentration proportioning was 5, 10 and 15%. Other procedures were the same with above.

Staining sample method (SS)

PCR products were mixed with 5 µL of diluted Gelred solution (1x, 3x) and Gelred stoste (10000x). Then 5 µL of mixture was electrophoresed in 1.5% gel without any dye (the other procedures were the same as above); finally, photos were taken (Huang et al.,2010).

Polyacrylamide gel electrophoresis (PAGE)

Immersion gels method (IG)

The proportion of PAGE gel was as follows: 60 ml of 6% polyacrylamide gel solution (29:1), 800 µL of 10% APS and 60 µL of TEMED. Then, the gel was cast at room temperature for 3 h to solidify. Next, 6 µL of PCR products were electrophoresed for 2 h at 180 V. Finally, the gel was put into staining solution to dye [the proportion of staining solution: addition of 10 µL of 10000X Gelred into 10 mL of NaCl solution (1 moL/L), then 1X TBE was used to make the total volume reach 100 mL], the gel was taken out after 30 min and photos were taken.

Precasting gels method (PG)

18 µL of Gelred (10000X) was added into 6% polyacrylamide gel solution (the proportion was the same as above). Then, 6 µL of PCR products were electrophoresed. Finally, photos were taken directly.

Staining sample method (SS)

PCR products were mixed with 5 µL of diluted Gelred solution (3X). Then, 6 µL of mixture was electrophoresed in 6% polyacrylamide gel (the proportion was the same as above), and photos were taken.

Orthogonal design of IG method for PAGE

There were three factors which influenced IG: The concentration of Gelred, the concentration of NaCl and immersion time. So, the authors did an orthogonal design of three factors and three levels-$L_9(3^4)$ (Tables 1 and 2).

RESULTS

Comparison of different Gelred methods used in AGE

Figure 1 is the comparison of two AGE methods with primer BPPCT023 and DNA of *A. cathayana* D. L.

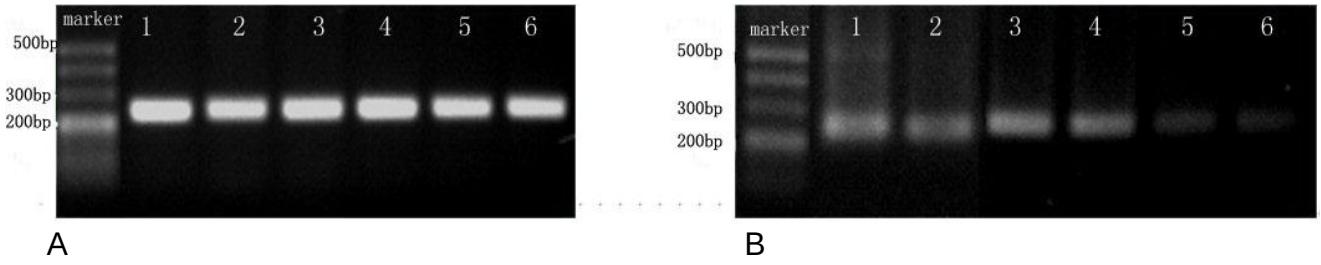

Figure 1. Comparison of two methods in agarose gel electrophoresis. A, Precasting gels method. B, staining sample method.

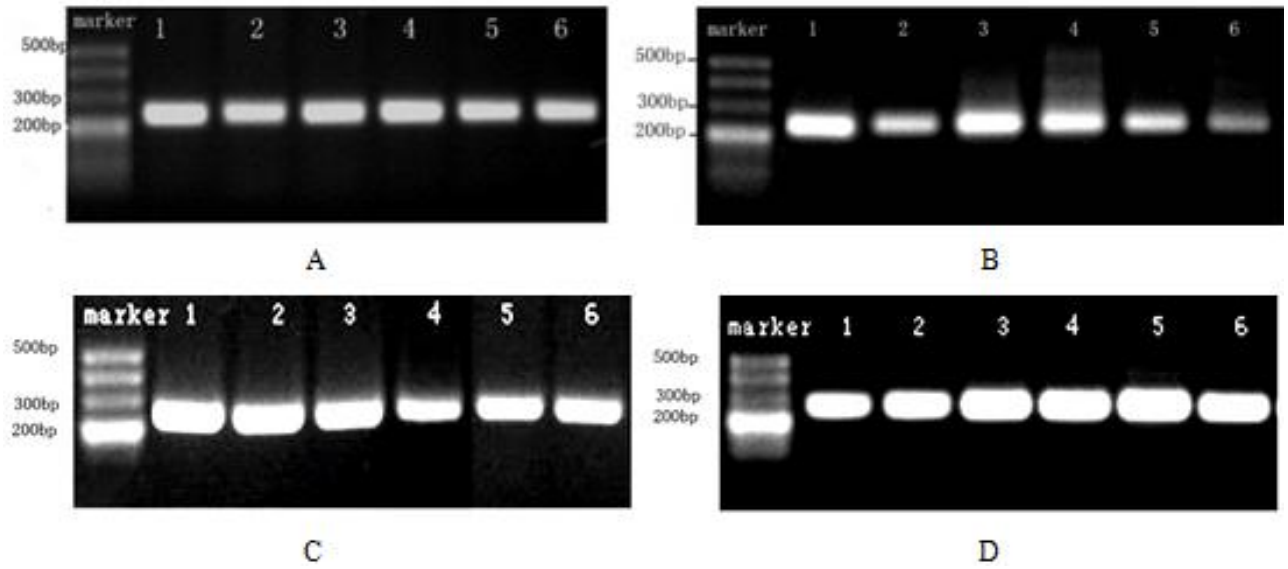

Figure 2. Results of different dyeing auxiliary concentrations. A, Gel without dying auxiliary. B, gel with 5% dying auxiliary. C, gel with 10% dying auxiliary. D, gel with 15% dying auxiliary.

(Fu et al., 2010) whose purpose bands' length is about 240 bp, concentration of Gelred is 1X and 3X. We can see from Figure 1 clearly, that there are marked differences between the two methods (Figure 1A and B). The bands from PG (A) are clear and bright, the background is clean too. By contrast, the bands colour from SS (B) is deep and the background is fuzzy. The reason for this phenomenon may be that mixture's uniformity was not very good or some air bubbles appeared in the process of mixing.

Optimization of PG method in AGE

In order to improve the effect of banding imaging for PG method, the authors used different concentration of dying auxiliary to do a gradient experiment. The results are shown in Figure 2. It can be obviously seen that bands in gel containing NaCl are brighter than the ones in gel without NaCl. The higher the concentration, the brighter the bands. However, even though bands are brightest when adding 15% NaCl, the bands are broadest as well, so that it will affect distinguishing different bands, therefore, it's best to add 5% NaCl into gels to improve banding brightness.

Optimization of SS method in AGE

Since SS method was the most widespread, the authors did an optimization for it with two different cultivars of *Armeniaca cathayana* D. L. (Fu et al., 2010). As shown in Figure 3, different dosage had different effect Figure 3B (product/dying = 1/1) had the best. Figure 3B shows that when products and dying have the same usage amount, concentration has significant influence on banding effect. In Figure 3B, the Gelred concentration of B1 and B2 was 1X, B3 and B4 was 3X, B5 and B6 was 10000X; we can see that the effects of B5 and B6 were worst, B3 and B4 were best, and the effects (B3,B4) were close to that of

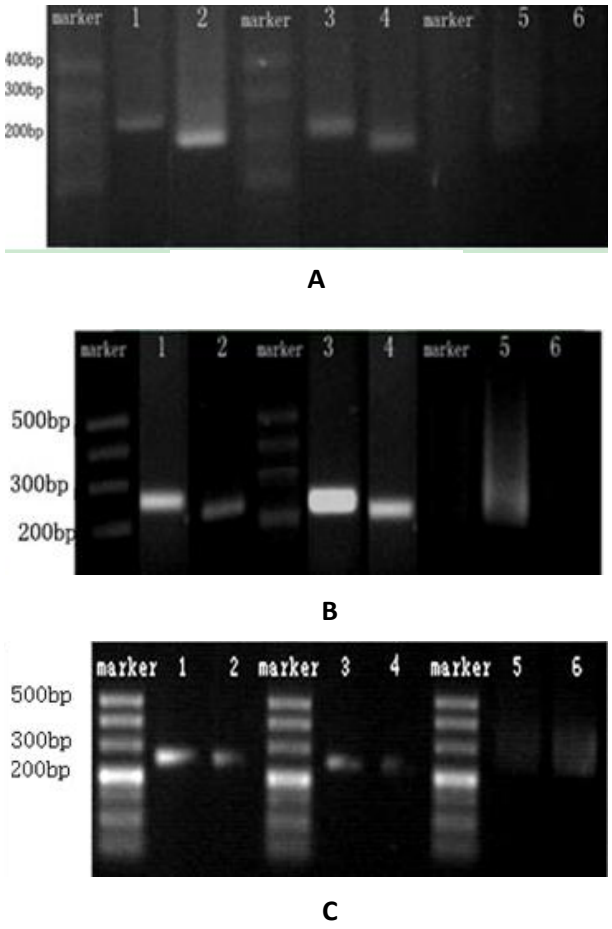

Figure 3. Comparison of different dosage by staining product. A, product (marker)/dying = 2:1. B, product (marker)/dying = 1:1. C, product/dying = 1-2 (marker without Gelred dying); 1-2 dying: 1XGelred, 3-4 dying: 3XGelred, 5-6 dying: Gelred stoste (10000x).

PG. In addition, when marker is without Gelred, the banding effect (C) is clearer than the others, so the marker contains dying itself, and it will have influence on banding effect if Gelred is used to re-dye.

Comparison of different Gelred methods used in PAGE

Figure 4 is the comparison of three PAGE methods with primer 7 and DNA of *A. cathayana* D. L. (Fu et al., 2010) whose purpose bands' length is about 250 to 300 bp and concentration of Gelred was 3X. Figure 4 shows that there was significant difference among the banding effects of IG (A), PG (B) and BSP(C), bands; Figure 4A are clear and bright, the background is also clean. In contrast, the band colour in other methods were much deeper, especially PG, and bands were almost hard seen.

In the PAGE experiments, banding effects of PG and SS were not very good; in order to confirm that it was for dying technology but not for other reasons, the authors used IG to re-dye the gel which was electrophoresed by SS for 30 min, then the two conditions were compared as shown in Figure 5. The unclear bands of Figure 5A were more distinct after re-dying. So, it shows that IG not only has good dying effect itself, but also can re-dye gels when other methods fail in dying, and it plays a role in the remedy.

Orthogonal experiment and analysis of PAGE for IG

IG is the best method for microsatellite PAGE banding. In order to enhance banding effect, the authors did an orthogonal design of three factors (concentration of Gelred, concentration of NaCl, immersion time) and three levels. According to bands' brightness and background, the authors divided results of orthogonal experiments (banding effects) into five levels, the best was 5, the worst was 1, (Table 2). Then, the author did a range analysis as shown in Table 3. Influence of 3 factors ranged from high to low in the order: immersion time, concentration of Gelred and concentration of NaCl.

In order to analyze further, the author did a variance analysis too (Table 4). The result was the same with range analysis; influence of immersion time reached significant level ($\alpha = 0.1$) and the other two factors did not reach significant level. Then, the authors did multiple comparisons for influence time. Results show that the third level (40 min) was the best; its banding effect reached significant level with the first one (20 min).

We can see from Table 4 that concentration of Gelred (1-3X) and NaCl (5-15%) did not have significant influence on banding effect. But if concentration of Gelred is too low, the band colour will be very deep; also, when the concentration of NaCl is too high, the background will be fuzzy. The best dispose of combination was NO.5: 2X Gelred, 10% NaCl and 40 min immersion.

DISCUSSION

Comparing two kinds of Gelred usage in AGE, it was seen that bands of PG were clear and bright, and the background was clean; so this method was the best. In addition, NaCl could enhance dying effect. The experiment of different NaCl concentration showed that higher concentration could enhance banding brightness, but at the same time, there were short comings in which background was fuzzy, bands were not too wide, etc. Even though PG was the best for AGE, gels were discarded after experiments, so this method wasted more dye and was more costly.

In the condition of products/3X dying = 1/1, the banding effect of SS was close to PG, but bands were not bright

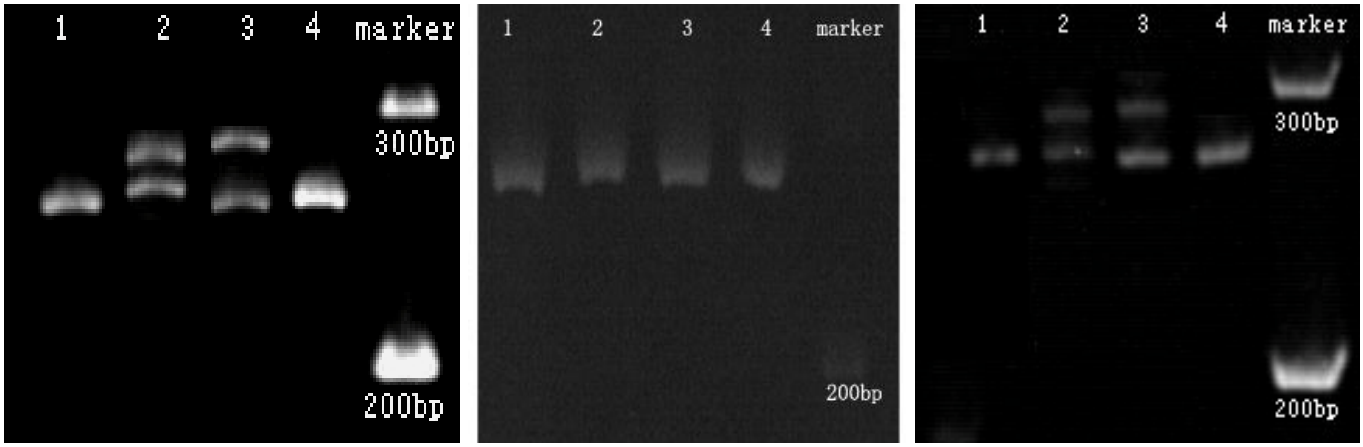

Figure 4. Results of different methods in PAGE. A, Immersion gels method; B, Precasting gels method. C, Staining sample method.

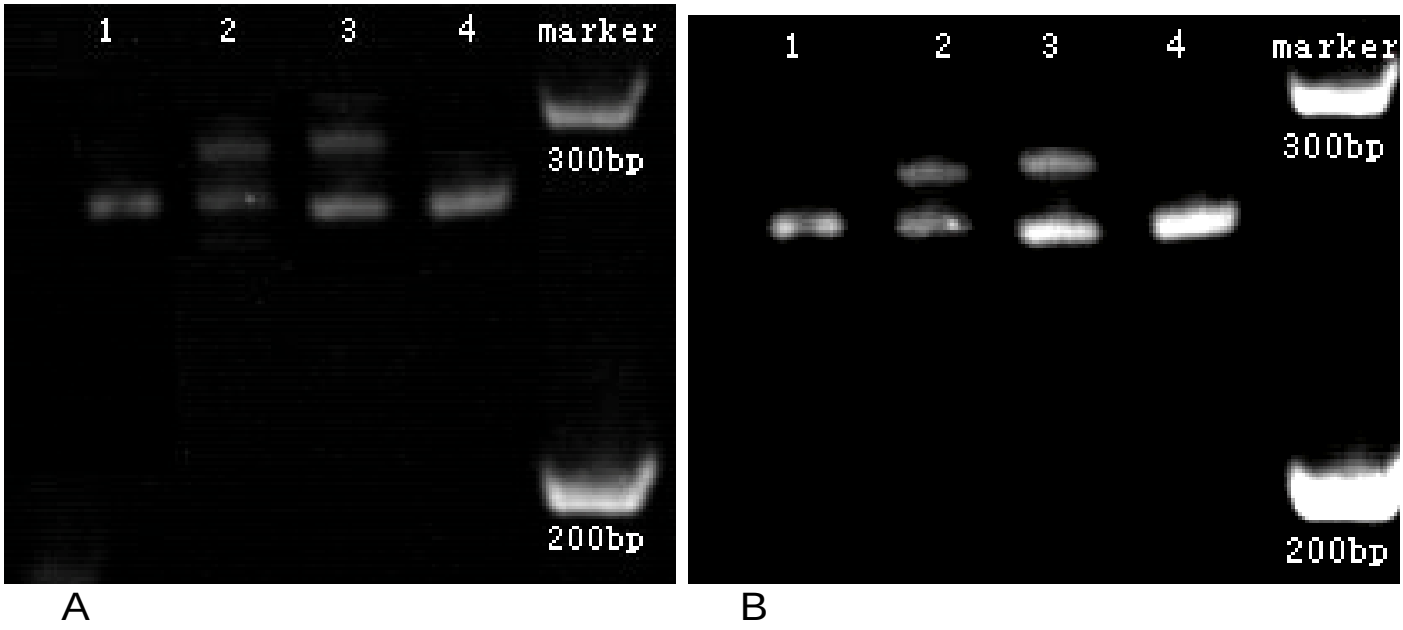

Figure 5. Comparison of banding results before and after dyeing with staining product. A, Banding effect before re-dying. B, banding effect after re-dying.

Table 3. Range analysis of orthogonal experiment.

Range analysis	Concentration of Gelred (X)	Concentration of NaCl (%)	Immersion time (min)
K_1	6.0	8.0	4.0
K_2	9.0	9.0	8.0
K_3	9.0	7.0	12.0
$K_1/3$	2.0	2.7	1.3
$K_2/3$	3.0	3.0	2.7
$K_3/3$	3.0	2.3	4.0
R	1.0	0.7	2.7

Table 4. Variance analysis for factors of orthogonal tests for dyeing by post gel staining.

Resource	SS	DF	MS	*F*	F_{α}
Concentration of Gelred	2.0	2	1.00	3.33	$F_{0.01}$=99
Concentration of NaCl	0.7	2	0.35	1.17	$F_{0.05}$=19
Immersion time	10.7	2	5.35	17.83*	$F_{0.1}$=9
Error	0.6	2	0.3		
Total	14	8			

and clear enough. In addition, if mixture was non-uniform or air bubbles appeared, bands were not ideal either. Finally, SS trivial steps were not suitable for a large sample of experiment. So, for AGE, PG method is better, and dying auxiliary can enhance banding effect.

The traditional imaging method of PAGE is argentation, but there are some short comings such as trivial steps and reagent toxicity. If the steps can be omitted, the experiments will be more safe and easy. The authors used three methods to optimize PAGE banding effect. The result showed that SS was not ideal, PG was limited by concentration and IG was the best method. Immersion time reached a significant level for banding effect in the orthogonal design experiment of IG, and the third level was the best, the other two did not reach significant level in the experiment, and disposing combination NO.5 (2X Gelred, 10% NaCl dying time 40 min) had the best banding effect.

Dying solution of IG can be repeatedly used for three to four times, so it is economical, and the process is very easy as well. Most importantly, when other methods fail in dying, IG can re-dye gels to help in banding. Therefore, it can be concluded that with regards PAGE, IG method is the best, and the method can re-dye gels of PG and SS.

Liu et al. (2011) put forward the new DNA band display technology of microsatellite, but there were some areas for improvement. In this study, the authors found better method for the DNA banding display technology through different experiments, and this lay a foundation for other studies.

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