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Assessment of silver-stained AFLP markers for studying DNA polymorphism in proso millet (*Panicum miliaceum* L.)

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ABSTRACT – (Assessment of silver-stained AFLP markers for studying DNA polymorphism in proso millet (*Panicum miliaceum* L.)). Proso millet (*Panicum miliaceum* L.) is a serious weed in North America. A high number of wild proso millet biotypes are known but the genetic basis of its phenotypic variation is poorly understood. In the present study, a non-radioactive silver staining method for PCR-Amplified Fragment Length Polymorphism (AFLP) was evaluated for studying genetic polymorphism in American proso millet biotypes. Twelve biotypes and eight primer combinations with two/three and three/three selective nucleotides were used. Pair of primers with two/three selective nucleotides produced the highest number of amplified DNA fragments, while pair of primers with three/three selective nucleotides were more effective for revealing more polymorphic DNA fragments. The two better primer combinations were EcoR-AAC/Mse-CTT and EcoR-ACT/Mse-CAA with seven and eleven polymorphic DNA fragments, respectively. In a total of 450 amplified fragments, at least 339 appeared well separated in a silver stained acrylamide gel and 39 polymorphic DNA bands were scored. The level of polymorphic DNA (11.5%) using only eight pairs of primers were effective for grouping proso millet biotypes in two clusters but insufficient for separating hybrid biotypes from wild and crop. Nevertheless, the present result indicates that silver stained AFLP markers could be a cheap and important tool for studying genetic relationships in proso millet.

Key words - AFLP, genetic diversity, molecular markers, proso-millet

RESUMO – (Avaliação de marcadores AFLP corados com prata para estudar polimorfísmo de DNA em proso millet (*Panicum miliaceum* L.)). Proso millet (*Panicum miliaceum* L.) constitui uma séria praga agrícola na America do Norte. Grande número de biótipos de proso millet selvagens são conhecidos mas a base genética da variabilidade fenotípica é pouco conhecida. No presente estudo foi avaliado o método não radioativo, de coloração com prata, para visualizar fragmentos polimóficos de DNA amplificados por PCR (AFLP) visando estudo de polimorfísmo genético em biótipos de proso millet americano. Foram usados doze biótipos e oito combinações de primers com dois/três e três/três nucleotídeos seletivos. Pares de "primers" com dois/três nucleotídeos seletivos produziram maior número de fragmentos de DNA, enquanto que pares de "primers" com três/três nucleotídeos seletivos foram mais efetivos para revelar maior número de fragmentos polimórficos de DNA. As duas melhores combinações de "primers" foram EcoR-AAC/Mse-CTT e EcoR-ACT/Mse-CAA, que produziram, sete e onze fragmentos polimórficos de DNA, respectivamente. Em um total de 450 fragmentos de DNA amplificados, pelo menos 339 apareceram bem resolvidos em gel de poliacrílamida corado com prata e 39 bandas polimórficas de DNA foram escrutinadas. O nível de polimorfísmo de DNA (11,5%) usando apenas oito combinações de "primers" foi efetivo para agrupar biótipos de proso millet em dois clusters, mas insuficiente para separar biótipos híbridos de biótipos selvagens e cultivados. Entretanto, o presente resultado indicou que o método de coloração com prata poderá ser ferramenta barata e importante para estudar relações filogenéticas em proso millet.

Palavras-chave - AFLP, diversidade genética, marcadores moleculares, proso millet

Introduction

Proso millet (*Panicum miliaceum* L.) is the major cultivated grain crop in Europe since 2000 BC (Anderson & Martin 1949, Grabouski 1971, Baltensperger 1996) and it is currently cultivated in Eastern Europe, Russia, China, India, and North America. This crop was first introduced into Canada in the 17th century and in the last 25 years, wild-proso millet has become one of the most aggressive grass weed in North America (Bough *et al*. 1986, Bough & Cavers 1987). High infestation by thos weed were first reported in Minnesota and Wisconsin in the early 1970s, and since this period, wild-proso millet has become a serious problem in different regions of the United States and Canada (Strand *et al*. 1973, WSSA 1992).

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Although it is considered to be a self-pollinated species, about 10% of cross pollination has been described in natural populations of proso millet (Colosi & Shaal 1997). Despite of many studies on phenotypic variation and ecological aspects in proso millet populations, their genetic basis was not adequately understood.

Studies applying molecular markers to access genetic variability and phylogenetic relationships in proso millet populations are still limited. In a study carried out by Colosi & Shaal (1997), within 398 individuals, 97 different genotypes were identified using RAPD markers, being 69 of them identified as wild, 26 crop and feral crop weed and 2 hybrids. In about 10% of the genotypes, DNA polymorphism suggested hybridization between wild and crop proso millet biotypes.

The Amplified Fragment Length Polymorphism (AFLP) technique (Vos *et al*. 1995, Blears *et al*. 1998) has been widely used for genetic studies in many organisms including cultivated plants and its wild relatives (Meksem *et al*. 1995, Cervera *et al*. 1996, Hillis *et al*. 1996, Tohme *et al*. 1996, Travis *et al*. 1996, Greef *et al*. 1997, Paul *et al*. 1997). Unfortunately, most of the procedures used to detect AFLP markers involve isotopes such as ^{32}P and ^{33}P that are relatively expensive, inconvenient to handle, and potential health hazard. In recent years, the development of non-radioactive methods based in silver stained gels (Cho *et al*. 1996, Vantoai *et al*. 1996) or the use of fluorophore labeled primers followed by separation of PCR-amplified products on an automated sequencer apparatus (Goulding *et al*. 2000, Mortimer & Arnold 2001) show many advantages over the use of radioisotopes. In both methods, the resolution is greater than that of the ^{32}P labelled gels and a larger number of better-defined bands could be scored. In comparison to other AFLP methods, the fluorescent amplified-fragment length polymorphism (FAFLP) technique showed a higher resolution of amplicons, thus increasing the number of distinguishable fragments (Arnold *et al*. 1999, Majeed *et al*. 2004, Kassama *et al*. 2006). However, FAFLP still has high start up costs and, for all but least heterogeneous species, fragment analysis requires software not yet freely available for every sequencer (Goulding *et al*. 2000). The silver staining method could be a choice to overcome these problems in laboratories that do not have those facilities.

The objective of this study was to evaluate the silver staining method to be used for studying genetic diversity of proso millet.

Material and methods

Three domesticated (South Dakota domestic brown, Colorado domestic white and Colorado domestic orange) and nine wild biotypes (Minnesota-Cambridge wild olive, Canada-Rosemount wild black, Oregon-Grand Island wild olive, Ontario Canada-Huron County wild black, Nebraska-Panhandle Center wild tan, Wyoming-Platte County wild brown, Colorado-Weld County wild black, Colorado-Weld County wild tan and Colorado wild white) of proso millet (*Panicum miliaceum* L.) were grown in a growth chamber with a 16 hours photoperiod, 25-20 °C (day-night temperature), and 50% relative humidity for two weeks. Eight plants of each biotypes were analyzed.

DNA was extracted from fresh leaves with DNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA). Leaves were ground with liquid nitrogen to obtain approximately 100 mg of fine powder. Cell debris and salt were precipitated using a QIAshredder spin column and the purified DNA was eluted with TE (Tris, EDTA) from a Dneasy spin column. The quality of DNA was checked in 1% agarose gel, and its concentration was measured by using a spectrophotometer.

The AFLP procedure (Vos *et al*. 1995) was based on AFLP™ Analysis System and AFLP Starter Primer protocol (Life Technologies,_Gaithersburg, MD) with some modifications. For restriction digestions, 500 ng of proso millet genomic DNA were digested to completion for 2 h and 30 min at 37 °C, with 2.5 units of *Eco*R I and *Mse* I restriction enzymes using 2 mL of the mix of *Eco*R I and *Mse* I, in buffer solution (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, 50% (v/v) glycerol, 0.1% Triton X-100). The restriction enzymes were inactivated by heating reaction at 70 °C for 15 min and immediately placed on ice for 5 min. Adapters ligation reactions were done with 24 µL of adapter ligation solution (5 pmoles *Eco*R I and 50 pmoles *Mse* I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate), 1 µL of 1 U μL^{-1} of T4 DNA ligase, and incubated at 20 °C for 2 h. A pre-amplification reaction was made mixing 5 µL of ligated DNA, 1 µL of 1 U μL^{-1} *Taq* DNA polymerase, 5 µL of 10 X PCR buffer, and 40 µL of pre-Amp primer mix, in a final volume of 51 µL. A 20 cycle (94 °C for 30 s, 52 °C for 60 s, 72 °C for 60 s) PCR was performed for pre-amplification of the DNA template. A dilution to 1:4 (v/v) of the pre-amplified product was made using TE buffer. All reagents and solutions were obtained in the AFLP Core reagents kit from Life Technologies. EcoR and Mse primers (30 ng each) were mixed with 2 µL of the diluted pre-amplified product, 2 µL of 10 X *Taq* buffer (200 mM Tris-HCl (pH 8.4), 15 mM $MgCl_2$, 500 mM KCl), 0.2 µL of 5 U μL^{-1} *Taq* DNA polymerase in a final volume of 20 µL. The selective amplifications were performed using the following two/three (EcoR-TC/Mse-CAA; EcoR-TG/Mse-CTC), and three/three pairs of primers (EcoR-AAG/Mse-CAA; EcoR-AGG/Mse-CTT; EcoR-AAG/Mse-CAG; EcoR-AGG/Mse-CTA; EcoR-AAC/Mse-CTT; EcoR-ACT/Mse-CAA). The PCR program was: one cycle at 94 ºC for 30 s; 65 ºC for 30 s; and 72 º for 60 s;

followed by a touch down phase of 13 cycles of 1 ºC lower annealing temperature each cycle. Afterwards, 23 cycles were performed at 94 ºC for 30 s; 56 ºC for 30 s; and 72 ºC for 60 s. The pre and selective amplifications were carried out in a PTC-100 programmable Thermal Controller (MJ Research, Inc, Waltham, MA).

An equal volume of formamide buffer (98% formamide, 10 mM EDTA, pH 8.0, 0,005% bromophenol blue, 0,005% xylene cyanol FF for tracking dye) was added to each PCR reaction. The samples were denatured by heating at 95 °C for 5 min and then, immediately placed on ice. Five microliters of each sample were loaded on the 5.3% polyacrylamide gel [acrylamide:bisacrylamide (20:1); 7.5 M Urea; 1X_TBE buffer]. Polyacrylamide gel electrophoresis was performed in a Sequi-Gen 0.04×38×50 cm apparatus (Bio-Rad Laboratories, Richmond, CA) in 1X TBE buffer at constant power (75 W) for 2 h or 2 h and 45 min at 50 °C with a PC 3000 Power Supply (Bio-Rad Laboratories, Richmount, CA). Samples were run until xylene cyanol was about 2-3 cm from the bottom of the gel.

After electrophoresis, gels were fixed with gentle agitation in 10% acetic acid for 15 min, followed by three 2 min water rinse. The silver staining was performed for 30 min in a solution containing 2 g silver nitrate; 3 mL of 37% formaldehyde in 2 liters water. Afterward, gels were rinsed in water for 10 s and stain was developed in a solution containing 60 g of sodium carbonate (Fisher Chemical); 3 mL formaldehyde; and 400 mL of sodium thiosulfate (Fisher Chemical). Once bands were visible, the developing process was stopped by adding 10% acetic acid to the staining solution.

Each polymorphic DNA band on gel was scored as an individual loci by presence (1) or absence (0). Genetic distances were calculated using the complement of the similarity index according to the equations 1 and 2:

$$Sij = 2a / (2a + b + c) \text{ (Nei \& Li, 1979)} \quad (1)$$

where *Sij* is the similarity index between two biotypes, *i* and *j*, *a* is the number of bands present in both *i* and *j* biotypes, *b* is the number of bands present only in the *i* biotype and c is the number of bands present only in the *j* biotype. Conversion to genetic distance (*Gdij*) was made by:

$$Gdij = 1 - Sij \quad (2)$$

The genetic distances were used for cluster analysis by dendrogram using the NTSYSpc, version 2.02 for windows (Exeter Software, Setauket, NY). The agglomeration criteria was the UPGMA (unweighted pair-group method with arithmetical averages) (Sneath & Sokal 1973). The confidence limits of UPGMA-based dendrogram was evaluated by using a 100 repetition bootstrap analysis (Hillis & Bull 1993) with GQMOL program (Cruz 1999).

Results and Discussion

A high resolution silver stained polyacrylamide gel without radioactive isotopes revealed to be an efficient technique to visualize AFLP markers in proso millet (figure 1). Silver-staining method shows many advantages in relation to standard radioactive procedure because it reduces time and costs as well as eliminates hazardous of working with radioisotopes (Vantoai *et al*. 1996, Chalhoulb *et al*. 1997). Eight combinations of AFLP primers were used for checking reproducibility and the resolution power of the silver staining method. At least 450 amplified fragments could be observed in polyacrilamide gels and some bands were difficult to be scored. Of the total bands, 339 were clearly separated on gels and 39 polymorphic DNA fragments (11.5%) were easily identified. Vos *et al*. (1995) reported that radiolabeled restriction fragments amplified by AFLP detect 50 to 100 fragments on a polyacrylamide gel. In the present study with proso millet an average of 56 amplified fragments were obtained per reaction, indicating that silver stained polyacrylamide gel retains the resolution power of the radiolabeled technique.

Although combination of primers with two and three selective nucleotides produced more amplified DNA fragments (figure 1A) than the combination of three and three selective nucleotides (figure 1B) those bands were difficult to be screened. Among primer combinations, EcoR-AAC/Mse-CTT and EcoR-ACT/Mse-CAA provided more information, producing seven and eleven polymorphic fragments, respectively (figure 1B, table 1). According to Blears *et al*. (1998), AFLP primers with only one or two selective nucleotide are necessary for revealing polymorphism in organisms with small genome size (10^6-10^7 bp), while additional selective nucleotides are required for those organisms with larger genomes (10^8-10^{10} bp). In the present study, results with proso millet, a species with small genome (10^6 bp), primers with two selective nucleotides were unable to reveal polymorphism between 12 biotypes (data not shown). On the other hand, primers combination with three selective nucleotides produced eight polymorphic fragments on average (figure 1B). The level of polymorphism per primer pair combination (11.5% on average) indicates that the AFLP may be useful for studying intraspecific variation and genetic relationships among proso millet biotypes.

UPGMA cluster analysis (Nei & Li 1979) using AFLP markers of twelve proso millet previously classified as wild and domesticated biotypes produced two groups without apparent relationships among wild and domesticated biotypes and was confirmed by the confidence test of bootstrap (figure 2). Six wild proso millet biotypes (Minnesota-Cambridge wild olive, Canada-Rosemount wild black, Oregon-Grand Island

wild olive, Colorado Weld County wild black, Colorado wild white and Ontario Canada-Huron County wild black) were clustered with domesticated plants. However, those biotypes could not be considered as standard of wild type since they show many phenotypic characters of domesticated plants like, non-dormancy and a relative high rate of radicle development during seed germination (Karam 2000). Previous studies considering ecophysiological traits (Striegel & Boldt 1981, Eberlein *et al*. 1990, Westra & Callan 1990, Karam 2000) concluded that all these six biotypes were intermediate between wild and domesticated populations with Minnesota Cambridge showing less weed characteristics than the other five wild biotypes.

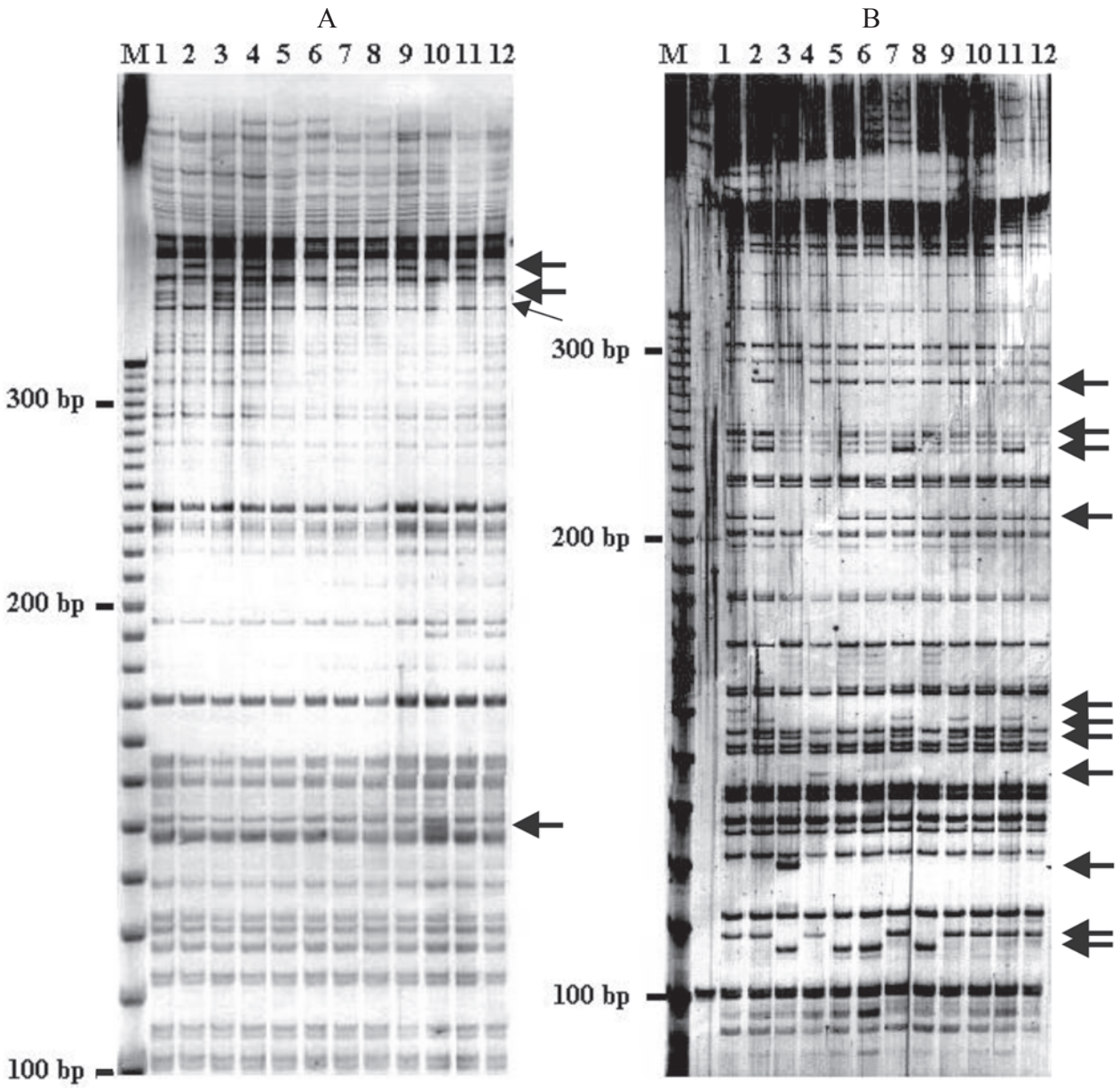

Figure 1. AFLP pattern of twelve proso millet (*Panicum miliaceum* L.) biotypes derived of the primer combination EcoR-TC/Mse-CAA (A) and EcoR-ACT/Mse-CAA (B). Numbers 1 to 12 on gel correspond to seeded biotypes: Minnesota-Cambridge wild olive; Canada-Rosemount wild black; Oregon-Grand Island wild olive; Ontario Canada-Huron County wild black; Nebraska-Panhandle Center wild tan; Wyoming-Platte County wild brown; Colorado-Weld County wild black; Colorado-Weld County wild tan; South Dakota domestic brown; Colorado domestic white; Colorado domestic orange, and Colorado wild white, respectively. Polymorphic bands are indicated by arrows. M = a 10 bp molecular marker (Invitrogen co., Carlsbad, Ca).

Table 1. Primer combinations of AFLP reactions and genetic diversity indexes for wild and domesticated proso millet (*Panicum miliaceum* L.) biotypes.

Primer combination	Total number of bands	Polymorphic bands		Genotype unique bands	
		Number	%	Number	%
EcoR-TC/Mse-CAA	54	4	7.41	1	25.0
EcoR-TG/Mse-CTC	56	0	–	0	–
EcoR-AAG/Mse-CAA	37	5	13.51	0	–
EcoR-AGG/Mse-CTT	41	4	9.76	1	25.0
EcoR-AAG/Mse-CAG	43	4	9.30	1	33.3
EcoR-AGG/Mse-CTA	34	4	11.76	2	50.0
EcoR-AAC/Mse-CTT	39	7	17.95	3	42.86
EcoR-ACT/Mse-CAA	35	11	31.43	3	27.27
Total	339	39	–	11	–

Afterwards, RAPD analysis (Colosi & Schaal 1997) demonstrated that Minnesota Cambridge was a genetically intermediary between wild and domesticated biotypes and probably originated by hybridization among them.

Based in AFLP markers, two wild biotypes (Minnesota-Cambridge wild olive and Ontario Canada-Huron County wild black) and one domesticated biotype (Colorado domestic orange) are genetically intermediary between wild (group two) and other domesticated biotypes in group one. The data suggest that hybridization of these three biotypes with wild and domesticated proso millet could be a more recent event compared with other members of group one (Canada-Rosemount wild black, Oregon-Grand Island wild olive, Colorado-Weld County wild black, Colorado wild white, Colorado domestic white and South Dakota domestic brown). The second AFLP group was constituted by Nebraska-Panhandle Center wild tan, Colorado-Weld County wild tan and Wyoming-Platte County wild brown. Studies based on phenotypic observations concluded that these biotypes were wild types without any trace of cultivated plants and may represent genuine wild proso millet biotypes (Karam 2000).

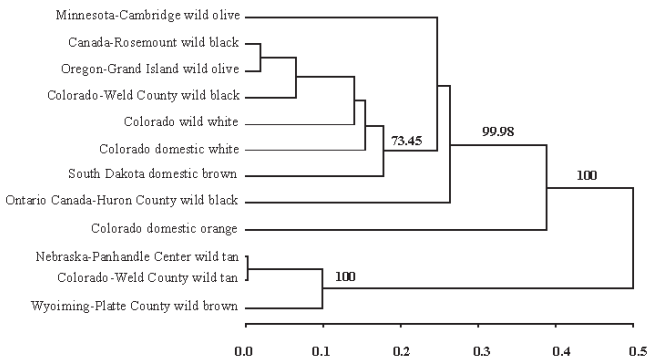

Figure 2. Dendrogram reporting AFLP-based genetic relationships among twelve proso millet (*Panicum miliaceum* L.) biotypes. The length of horizontal branches are proportional to genetic distance among biotypes while vertical branches are arbitrary. Numbers in *x*-axis correspond to genetic distances and values on branches indicate the number of times (expressed as percentage) that genotypes were joined together with a 100 cycles of bootstrap analysis (Cruz 1999). Dendrogram was generated with NTSYSpc software, version 2.2 (Exeter Software co., Setauket, NY).

The present result with AFLP markers is in accordance with previous studies in proso millet using RAPD markers (Colosi & Schaal 1997) and phenotypic parameters (Striegel & Boldt 1981, Eberlein *et al*. 1990, Westra & Callan 1990, Karam 2000) and demonstrated the usefulness of silver staining method for revealing AFLP markers for studying genetic diversity in proso millet.

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