NOTE

Species identification in seven small millet species using polymerase chain reaction – restriction fragment length polymorphism of *trn*S-*psb*C gene region

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Abstract: The chloroplast *trn*S-*psb*C gene regions from total genomic DNA of 119 accessions from seven small millet species were amplified by polymerase chain reaction (PCR) and digested with eight restriction enzymes individually as well as in combinations of two enzymes to generate restriction fragment length polymorphism (PCR–RFLP). PCR–RFLP with individual enzymes revealed polymorphism between only some species. However, all the species could be distinguished by using a combination of two enzymes, specifically *Hae*III and *Msp*I. PCR–RFLP of 11 to 20 accessions with the same enzyme combination showed no intraspecific variation, which established that the differential banding patterns were species specific. In contrast, the same enzyme combination was not useful for differentiating different species of the genera *Cajanus, Rhyncosia*, *Abies, Rhizophora, Ceriops,* and *Bruguiera,* and it also revealed intraspecies variation in three species of *Abies*. The present study indicated that digestion of *trn*S-*psb*C with two four-base recognizing enzymes reveals more variation than with either enzyme alone and that it may be a method of choice for species identification in some genera.

Key words: small millets, PCR–RFLP, *trn*S-*psb*C, chloroplast DNA.

Résumé : La région chloroplastique *trn*S-*psb*C a été amplifiée par la réaction de polymérisation en chaîne (PCR) à partir de l'ADN génomique total chez 119 accessions de sept espèces de millets à petits grains. Ces produits d'amplification ont été digérés à l'aide de huit enzymes de restriction, employées individuellement ou en paires, afin de révéler du polymorphisme de longueur des fragments de restriction (PCR–RFLP). L'emploi d'une seule enzyme à la fois permettait de révéler du polymorphisme uniquement entre certaines espèces. Cependant, toutes les espèces pouvaient être distinguées en utilisant une combinaison de deux enzymes : *Hae*III et *Msp*I. Une analyse PCR–RFLP de 11 à 20 accessions à l'aide de la même combinaison enzymatique n'a révélé aucun polymorphisme intraspécifique démontrant que les différents profils obtenus étaient spécifiques de chaque espèce. Par contre, la même combinaison d'enzymes n'a pas permis de distinguer différentes espèces appartenant aux genres *Cajanus*, *Rhyncosia*, *Abies*, *Rhizophora*, *Ceriops* et *Bruguiera* en plus de révéler de la variation intraspécifique au sein de trois espèces du genre *Abies*. Cette étude indique que la digestion de la région *trn*S-*psb*C avec deux enzymes reconnaissant des sites tétranucléotidiques révèle plus de variation que chacune des deux enzymes prises individuellement et qu'une telle approche pourrait s'avérer une méthode de choix pour l'identification des espèces chez certains genres.

Mots clés : millets à petits grains, PCR–RFLP, *trn*S-*psb*C, ADN chloroplastique.

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Introduction

Minor millets or small millets are the small-grained cereals belonging to the family Poaceae. The First International Small Millet Workshop reported 35 species of grasses from 20 genera as small millets (de Wet 1986) and it was noted that two-thirds of that number were no longer being cultivated (Doggett 1993). The most important small millet is finger millet, which is distributed in eastern Africa and South Asia, particularly India (Hilu and de Wet 1976). Foxtail and proso millets are grown across temperate Eurasia extending into the semi-arid tropical Asia. Banyard millet is cultivated in China, Korea, and Japan. Sawa millet is grown in Central and South India (de Wet 1986). Kodo millet is cultivated in India and Bangladesh (de Wet et al. 1983). Little millet is grown in the Eastern Ghats of India (de Wet 1986).

International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India, possesses the largest collection of 7082 accessions, belonging to seven species of small millets collected from 42 countries. Classification of these accessions based on morphological characters was carried out in collaboration with Crop Evolution Laboratory, University of Illinois, U.S.A. (Prasada Rao et al. 1993). Assessment of genome origin and estimation of genetic diversity in the genus *Eleusine* was done using random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and interspersed simple sequence repeat (ISSR) markers (Salimath et al. 1995; Hilu 1995). Apart from this, there is no worthwhile information on application of molecular markers in small millets. We have analysed intraspecific genetic diversity in seven small millet species using RAPD and RFLP markers, and genome relationships among these species using amplified fragment length polymorphism (AFLP) and RFLP markers that are being published elsewhere. The present report communicates the development of simple polymerase chain reaction (PCR) based species-specific markers for unambiguous identification of seven small millet species. In this method, a particular gene is amplified by universal primer pairs designed from conserved sequences and subsequently digested with restriction enzymes to reveal restriction fragment length polymorphism in PCR-amplified fragments (PCR–RFLP). RFLP and PCR–RFLP markers have proven to be reliable for genetic studies in many species (Wang and Szmidt 1994; Tsumura et al. 1995). In general, due to its uniparental inheritance, and to the fact that it does not undergo recombination, the inheritance of the chloroplast genome is linear over generations. The slow rate of sequence and structural evolution of this genome (Palmer 1987; Birky 1988) provides opportunities for identifying species diagnostic markers (Parducci and Szmidt 1999; Parani et al. 2000).

Materials and methods

For the present study, 119 accessions from seven small millet species were obtained from ICRISAT as seeds (Table 1). The seeds were germinated in pots, and leaf tissue from 15-days-old-seedlings were used for DNA isolation. The source and description of the samples from *Abies, Cajanus, Rhyncosia, Rhizophora, Ceriops, and Bruguiera* used in this study (Table 2) were described previously (Parducci and Szmidt 1999; Lakshmi 1999; Lakshmi et al. 2000)

DNA isolation

Five grams of tissue was ground under liquid nitrogen, and suspended in a buffer containing 100 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA and 0.5% Triton-X 100. The suspension was centrifuged at 5000 × *g* for 10 min at 4°C. The pellet was suspended in 2% cetyltrimethylammonium bromide (CTAB) buffer (Saghai-Maroof et al. 1984) and incubated at 60°C for 30 min. The DNA was extracted with chloroform – isoamyl alcohol (24:1) and precipitated with 0.6 volume of ice-cold isopropanol. The pellet containing DNA was dissolved in TE buffer and purified from RNase and proteins following standard procedures (Sambrook et al. 1989).

PCR amplification of *trn*S-*psb*C

The chloroplast gene region *trn*S-*psb*C was PCR amplified using a pair of universal primers; primer 1: 5′-GGT TCG AAT CCC TCT CTC TC-3′, primer 2: 5′-GGT CGT GAC CAA GAA ACC AC-3′ (Demesure et al. 1995) as described before (Parani et al. 2000). The temperature profile consisted of a total of 35 cycles with 1 min (4 min for the first cycle) at 94°C for template denaturation, 1 min at 63°C for primer annealing, and 2 min (15 min for the final cycle) at 72°C for primer extension. The amplified products were precipitated with ice-cold ethanol, washed with 70% ethanol, and dissolved in TE buffer. The DNA was quantified by agarose gel electrophoresis, and ethidium bromide staining.

PCR–RFLP of *trn*S-*psb*C

About 200 ng of *trn*S-*psb*C in 10 μL reaction mix was digested to completion according to the manufacturer's instructions. Eight four-base-recognizing restriction enzymes viz. *Hae*III, *Msp*I, *Rsa*I, *Sau*3AI, *Taq*I, *Alu*I, *Acc*II, and *Hha*I were used individually as well as in combination of *Hae*III with the remaining seven enzymes (double digestion). The restricted products were separated on 10–15% native acrylamide gels and stained with ethidium bromide (Sambrook et al. 1989).

Results and discussion

To survey for the restriction sites, the *trn*S-*psb*C gene region in one accession from each of the seven millet species was amplified by PCR and purified as described above. Amplification at 63°C produced a 1.6 kb fragment in all the species as expected, and there was no size variation among the species analysed. Digestion of purified *trn*S-*psb*C with the eight restriction enzymes individually showed the presence of at least two restriction sites for each enzyme. This is in contrast with earlier reports in the species of *Abies* (Ziegenhagen et al. 1995; Ziegenhagen and Fladung 1997), and *Cajanus* and its allied genera (Lakshmi et al. 2000) where many enzymes did not have sites at all.

Although the PCR–RFLP with single enzymes detected interspecific polymorphism (except for *Taq*I) it could not differentiate all the species. Therefore, *Hae*III was combined with each of the other seven enzymes to carry out double digestion. This increased the number of restriction products in each species to a minimum of six (from two in single digestion). The number of polymorphic fragments across the seven species increased considerably with each combination of enzymes, and it ranged between 8 and 12. As a result, 74 polymorphic loci could be obtained from seven double digestions, and that showed a definite increase in the resolu-

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Table 1. List of small millet accessions used in the present study.

Minor millet	Species	Subspecies	Race	Accession No. (ICRISAT)
Finger millet (20)	*Eleusine coracana*	*coracana*	*elongata*	IE 7, 24, 503, 798, 801
			plana	IE 771, 2197, 2297, 2301, 2303
			compacta	IE 2001, 2185, 2256, 2286, 2836
			vulgaris	IE 4, 6, 23, 363, 372
Foxtail millet (15)	*Setaria italica*	*italica*	*moharia*	ISe 276, 361, 403, 429, 456
			maxima	ISe 738, 739, 740, 741, 759
			indica	ISe 2, 3, 8, 15, 17
Kodo millet (15)	*Paspalum scrobiculatum*		*regularis*	IPS 318, 320, 325, 326, 329
			irregularis	IPS 319, 321, 328, 583, 50
			variabilis	IPS 48, 49, 62, 70, 77
Proso millet (20)	*Panicum miliaceum*	*miliaceum*	*patentissium*	IPM 9, 16, 33, 43, 102
			contractum	IPM 19, 27, 65, 66, 256
			compactum	IPM 296, 1582, 1584, 1588, 1598
			ovatum	IPM 104, 105, 2026, 2027, 2069
Little millet (11)	*Panicum sumatrense*	*sumatrense*	*nana*	IPMR 3, 5, 369, 447, 449, 706
			robusta	IPMR 452, 702, 703, 704, 710
Sawa millet (19)	*Echinochloa colona*	*frumentacea*	*stolonifera*	IEC 98, 103, 104, 105, 133
			intermedia	IEC 53, 54, 55, 56, 57
			robusta	IEC 52, 59, 134, 135
			laxa	IEC 126, 127, 130, 131, 289
Banyard millet (19)	*Echinochloa crusgalli*	*crusgalli*	*crusgalli*	IEC 405, 410, 413, 416
			macrocarpa	IEC 400, 329, 330, 331, 322
			utilis	IEC 436, 493, 502, 506, 509
			intermedia	IEC 511, 513, 515, 517, 520

Note: Figures given in parentheses indicate the number of accessions used from the respective species.

Table 2. List of *Cajanus, Rhyncosia, Abies*, and Rhizophoraceae species used in the present study. One accession was used from each species, excepting *A. alba*, *A. cephalonica*, and *A. numidica* in which the number of accessions used are given in parentheses.

Cajanus	*Rhyncosia*	*Abies*	Rhizophoraceae
C rugosus	*R. rohtii*	*A. alba* (4)	*Ceriops decandra*
C. marmoratus	*R. densiflora*	*A. bornmulleriana*	*C. tagal*
C. goensis	*R. minima*	*A. cephalonica* var. *cephalonica* (4)	*Bruguiera cylindrica*
C. grandiflorus	*R. totta*	*A. cilicica*	*B. gymnorhhiza*
C. cinereus	*R. pyramidalis*	*A. concolor* var. *concolor*	*B. parviflora*
C. plurifora	*R. hirta*	*A. nebrodensis*	
C. scarabaeoides		*A. nordmanniana*	
C. lineatus		*A. numidica* (3)	
C. cajanifolius		*A. pinsapo* var. *marocana*	
C. platycarpa			
C. albicans			
C. cajan			
C. mollis			
C. reticulatus			
C. sericeus			

tion of sequence variations between the chloroplast genes from the seven millet species.

Among the enzyme combinations used, *Hae*III and *Msp*I (double digestion) was able to distinguish all the species unambiguously (Fig. 1A). These were the two enzymes that showed maximum polymorphism when used individually also. This combination of enzymes is convenient to use in the sense that both are compatible for digestion in single buffer supplied by most of the manufacturers. These enzymes were also found to digest the DNA to completion without the need to purify the PCR product. Standardization of the percentage of acrylamide gels was found to be essential so as to clearly separate the majority of the restriction products. Good resolution was achieved in 13% gels for the double digestion with *Hae*III and *Msp*I. Therefore, we amplified the *trn*S-*psb*C gene region from 119 accessions (Table 1), directly digested with *Hae*III and *Msp*I, and resolved in 13% acrylamide gels. The results showed perfect conser-

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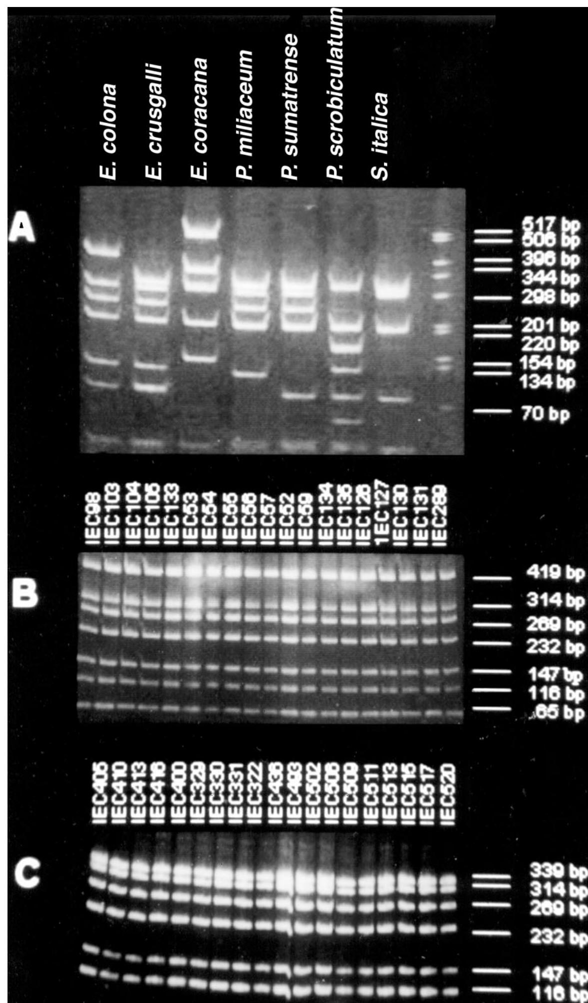
Fig. 1. Double digestion of *trn*S-*psb*C gene region amplified by PCR from one representative accession from each of the seven small millet species (1A), 19 accessions of *Echinochloa colona,* and 18 accessions of *E. crusgalli* with the restriction enzymes *Hae*III and *Msp*I. The digested products were resolved in 13% native acrylamide gels and stained with ethidium bromide. The 1 kb ladder DNA marker (Gibco-BRL) was used for calculating the size of restriction fragments.



vation of the restriction sites among all the accessions within each species. The restriction banding pattern of *trn*S-*psb*C from 19 accessions of *Echinochloa colona* and *E. crusgally* after double digestion with *Hae*III and *Msp*I are shown in Fig. 1B and 1C. This clearly indicated that the observed interspecific differential banding patterns were species specific.

For species identification we require a marker system that is variable enough to distinguish all the species of a genus but conserved enough not to show any variation within each species. Moreover, different applications of species-specific markers require that the techniques involved to resolve such markers are simple and inexpensive. Several chloroplast genes could be amplified by a simple PCR method as the primer binding sites are conserved, and digested with several restriction enzymes to reveal polymorphism within the genes (PCR–RFLP). This was reported to be applicable only above species level (Ziegenhagen et al. 1995) due to lack of variation. However, in our earlier studies on mangrove and mangrove associate species we have found that PCR–RFLP of *trn*S-*psb*C with *Hae*III enzyme showed significant levels of polymorphism at the species level itself. Interspecific variations were found in three genera viz. *Rhizophora, Avicennia*, and *Suaeda*. Species-specific restriction patterns were found in *Rhizophora* and *Suaeda* (Parani et al. 2000). In another study on the genus *Abies* (Parducci and Szmidt 1999), species diagnostic markers were observed in 4 of the 10 species by analysing 10 PCR-amplified chloroplast DNA regions after digestion with several restriction enzymes. Therefore, we presumed that species-specific markers could be developed for other genera either (*i*) by using multiple enzymes for restriction or (*ii*) by amplifying large fragments of chloroplast genome.

In the present study, the first approach proved to be useful in case of millet species. However, the same approach was not successful with 15 species of *Cajanus,* 6 species of *Rhyncosia*, 9 species of *Abies,* 2 species of *Ceriops*, and 3 species of *Bruguiera* from the mangrove family Rhizophoraceae (Table 2). Although digestion with *Hae*III and *Msp*I yielded species-specific profiles in small millets, it showed monomorphic pattern across all the 15 species of *Cajanus* and 6 species of *Rhyncosia* used in the present study (data not shown). Differentiation of all the species of *Abies* was not possible even by using certain combinations of three enzymes (e.g., *Msp*I, *Hae*III, and *Sau*3AI), and there were intraspecific variations in *A. alba*, *A. cephalonica,* and *A. numidica* (data not shown). Intraspecific variations in *trn*S-*psb*C after digestion with single restriction enzymes were observed earlier in *A. alba* (Ziegenhagen et al. 1995; Parducci and Szmidt 1999) and also in *A. cephalonica* and *A. numidica* (Parducci and Szmidt 1999). Therefore, additional chloroplast genes are being analyzed for developing species-specific markers in these species.

PCR–RFLP is a cost-effective method for surveying many samples for variations in chloroplast DNA regions. In the present study, double digestion of the PCR amplified chloroplast gene region *trn*S-*psb*C with four-base-recognizing restriction enzymes followed by acrylamide gel electrophoresis and staining with ethidium bromide showed discrete banding patterns that were useful for analyzing interspecific variations. This method with a particular enzyme combination (*Hae*III and *Msp*I) revealed species-specific markers for seven small millet species. This will be useful in genetic studies, species identification, parentage determination of inter-specific hybrids, analysis of species admixtures, etc. However, the present study also indicated that the application of this technique for developing species-specific makers in other genera should be evaluated on a case-by-case basis.

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References

Birky, C.W. 1988. Evolution and variation in plant chloroplast and mitochondrial genomes. *In* Plant Evolutionary Biology. *Edited by* L. Gottlieb and S. Jain. Chapman and Hall, London. pp. 23–53.

Demesure, B., Sodzi, N., and Petit, R.J. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. **4**: 129–131.

de Wet, J.M.J. 1986. Origin, evolution and systematics of minor cereals. *In* Small millets in global agriculture. *Edited by* A. Seetharam, K.W. Riley, and G. Harinarayana. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, India.

de Wet, J.M.J., Prasada Rao, K.E., Mengesha, M. H., and Brink, D.E. 1983. Diversity in kodo millet, *Paspalum scrobiculatum*. Econ. Bot. **37**: 159–163.

Doggett, H. 1993. Introduction. *In* Advances in small millets. *Edited by* K.W. Riley, S.C. Gupta, A. Seetharam, and J.N. Mushonga. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, India. pp. 3–8.

Hilu, K.W. 1995. Evolution of finger millet: Evidence from random amplified polymorphic DNA. Genome, **38**: 232–238.

Hilu, K.W., and de Wet, J.M.J. 1976. Domestication of *Eleusine coracana* (L.) Gaertn. Econ. Bot. **30**: 199–208.

Lakshmi, M. 1999. Genetic diversity in mangrove species of the family Rhizophoraceae. Ph.D. thesis, M.S. Swaminathan Research Foundation, Chennai, India.

Lakshmi, M., Senthilkumar, P., Parani, M., Jithesh, M.N., and Parida, A. 2000. PCR-RFLP analysis of chloroplast gene regions in *Cajanus* (Leguminosae) and allied genera. Euphytica, **116**: 243–250.

Palmer, J.D. 1987. Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am. Nat. **130**: S6–S29.

Parani, M., Lakshmi, M., Ziegenhagen, B., Fladung, M., Senthilkumar, P., and Parida A. 2000. Molecular Phylogeny of Mangroves VII. PCR-RFLP of *trn*S-*psb*C and *rbc*L gene regions in 24 mangrove and mangrove associate species. Theor. Appl. Genet. **100**: 454–460.

Parducci, L., and Szmidt, A.E. 1999. PCR-RFLP of cpDNA in the genus *Abies.* Theor. Appl. Genet. **98**: 802–808.

Prasada Rao, K.E., de Wet, J.M.J., Gopal Reddy, V., and Mengesha, M.H. 1993. Diversity in the small millets collection at ICRISAT. *In* Advances in small millets. *Edited by* K.W. Riley, S.C. Gupta, A. Seetharam, and J.N. Mushonga. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, India.

Saghai-Maroof, M.A., Soliman, K.M., Jorsenson, R.A., and Allard, R.W. 1984. Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. U.S.A. **81**: 8014–8018.

Salimath, S.S., de Oliveira, A.C., Godwin, I.D., and Bennetzen, J.L. 1995. Assessment of genome origin and genetic diversity in the genus *Eleusine* with DNA markers. Genome, **38**: 757–763.

Sambrook, J., Fritsch, F.F., and Maniatis, T. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Tsumura, Y., Yoshimura, K., Tomaru, N., and Ohba, K. 1995. Molecular phylogeny of conifers using RFLP analysis of PCR amplified specific chloroplast genes. Theor. Appl. Genet. **91**: 1222–1236.

Wang, X.R., and Szmidt, A.E. 1994. Hybridisation and chlorplast variation in a *Pinus* species complex from Asia. Evolution, **48**: 1020–1031.

Ziegenhagen, B., and Fladung, M. 1997. Variation in the *psb*C gene region of gymnosperms and angiosperms as detected by a single site polymorphism. Theor. Appl. Genet. **94**: 1065–1071.

Ziegenhagen, B., Kormutak, A., Schauerte, M., and Scholz, F. 1995. Restriction site polymorphism in chloroplast DNA of silver fir (*Abies alba* Mill.) For. Genet. **2**: 99–107.

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