

EFFECTS OF DNA EXTRACTION PROCEDURES ON THE CHARACTERIZATION OF A MINOR TUBER CROP 'RIZGA' (*PLECTRANTHUS ESCULENTUS* N. BR.)

P. M. KYESMU and S. H. MANTELL

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ABSTRACT

Effects of DNA extraction procedures on the characterization of a minor tuber crop 'Rizga' (*Plectranthus esculentus* N.E.Br.) was demonstrated. Results indicated that evaluation of DNA extraction procedures was necessary in developing a standard protocol that would be suitable for the DNA fingerprinting of *P.esculentus* clones. Whereas DNA polymorphism was observed in clones under evaluation when extraction procedure DEx1 was used, there was non observed in the case of DEx2. Furthermore, the estimation of the genetic distances and similarities between some clones of *P.esculentus* using RAPD profiles obtained from DEx1 procedure indicated that clones VTH and VTHc were similar with a coefficient of 0.83. Clones LK and VTJ had a coefficient of 0.67 followed by VTB and LM with a coefficient of 0.57 as also VTA and VTF with a coefficient of 0.57. Coefficients of the other clones under study are further discussed.

Keywords: Characterization, RAPD, tuber, *Plectranthus esculentus*

INTRODUCTION

All biological objects possess a unique genetic signature that can be visualised using either DNA or Isozyme techniques. *Plectranthus esculentus* being biological objects possess characteristic phenotypes or physical appearances because of their unique hereditary compositions. The principle of individual uniqueness within all tissues of the same plant provides the basis for genetic profiling. These signatures-'fingerprints' or profiles (as they are often called), have broad applications in plant sciences and the process of evaluating the profiles in biological objects is viewed as **genetic fingerprinting**: that is the characterisation of one or more relatively rare features of the plant genome or its hereditary makeup (Kirby 1992).

Isozymes were the first molecular markers to be used in plant breeding programmes for cultivar characterisation, but the presence or absence of small amounts of information (bands) observed in some systems regarding their variations and genetic natures, limits their usefulness. The use of isozymes as genetic markers is based on the fact that these are direct products of genes and are a function of both DNA quality and the expression of the phenotypic characters, in view of the biochemical pathway leading to the formation of a certain phenotype (Dias

et al., 1994). Some of this information does not completely discriminate varieties (Kyesmu and Mantell, 1999). This has, therefore, led to the widespread use of polymerase chain reaction (PCR) marker techniques using random primers. The use of random primers led to the term "RAPD" coined by Williams *et al* (1990). The technique, unlike isozymes and structural proteins, allows for the detection of variations at the DNA level. It is quick, simple, efficient and more significantly, can be detected using small amounts of DNA (10ng per reaction) and any tissue at any developmental stage.

The application of this technique in the discrimination of not only plant cultivars, but also in the characterisation of species and genera has been widespread and is currently considered the fastest and the most generally accepted technique in the genetic fingerprinting of organisms. For example, cultivar-specific DNA profiles in rye were revealed by PCR using randomly amplified polymorphic DNA (RAPD) sequences. Ten-mer oligonucleotide primers were used for the amplification of genomic DNA of the rye cultivars and not all primers revealed polymorphism (Iqbal and Lane-Rayburn, 1994). In another experiment, Keil and Griffin (1994) working on *Eucalyptus* spp observed RAPD profiles that are unique to a genotype and can be generated reliably

P. M. Kyesmu, Department of Botany, Faculty of Natural Sciences, P. M.B. 2084, University of Jos, Jos, Nigeria.

S. H. MANTELL, Unit for Advanced Propagation Systems (UAPS), Department of Agriculture, Horticulture and the Environmental Wye College, University of London, Wye, Ashford, KENT TN25 5AH, UK.

and simply such that even closely related genotypes can be distinguished. Furthermore, Barcaccia *et al.* (1994) while working on alfalfa meiotic mutants observed that the use of oligonucleotide (ten-base-long nucleotide) primers differed significantly in their capacity for detecting polymorphism, though the PCR-based characterisation was used to determine whether or not the apomeiotic diploid eggs of alfalfa developed through parthenogenesis.

Plectranthus esculentus is a minor tuber crop that belongs to the mint family (Labiatae) and is cultivated mainly for its edible tubers (Purseglove, 1987 and Rivera-Nunez and Oben de Castro, 1992). Its cultivation has been reported around West, Central and East Africa (Tindall, 1983). Being an under-utilised crop, it has never been characterised using any biochemical or DNA fingerprinting techniques, except that reported by us (Kyesmu and Mantell 1999, isozymes characterisation).

The objective of the current study was to principally assess DNA extraction procedures as they affect the detection of polymorphism in RAPD profiles of *P. esculentus* clones. This is expected to lead to the development of a suitable DNA fingerprinting technique for carrying out germplasm auditing and management functions in respect of 'Rizga' germplasm collections at some stage in future.

MATERIALS AND METHODS

Plant materials

Twelve *in vitro* donor clones of *P. esculentus*, culture procedures, media composition and culture conditions used in this study were similar to those described elsewhere (Kyesmu and Mantell, 1998). The clones used were collected from two different areas of the Jos Plateau (Vom-Turu and Langtang). Forty-two day old *in vitro* shoot cultures were used as materials for the analyses. Clones used were: VTA (A), VTB (B), VTD (D), VTF (F), VTG (G), VTH (H), VTHc (Hc), VTI (I), VTJ (J), LK (K), LM (M) and LN (N).

RAPD analyses

The method used for RAPD was essentially the same as that described and optimised by Aichitt *et al.* (1993) for his work on the development of DNA markers in date palm. During the course of the current investigation, several modifications of the technique were made and these are described in the following sections in detail.

Plant DNA extraction (DEX1)

The procedure used for DEX1 was a modified version of the method described by Kang *et al.* (1993). Plant tissues (root tips ca. 5 - 10 mm in length of 42days *in vitro* shoot cultures) were placed in a 1.5 ml Eppendorf tube with 50µl DNA extraction buffer consisting of 100mM Tris-HCl, 100mM EDTA and 2% SDS, pH 8.0 using a sterile alcohol flamed forceps. A home-made homogenizer [made by moulding the heat-softened narrow end of a 1.5-ml blue pipette tip (Gilson, UK) to fit tightly into the bottom of a 1.5-ml Eppendorf tube] was used to homogenise the tissues and to release the total DNA into the buffer. The extract was cleared by centrifugation in a micro-centrifuge (with a 12 x 1.5 ml rotor, model 32785-108) at 11,600g for 30s. One µl aliquot of the supernatant was diluted in 1000µl 10mM Tris to obtain a 1:1000 dilution of the original solution. One µl of this diluted solution was used in a 33µl PCR reaction (Table 1)

Plant DNA extraction (DEX2)

The procedure used for DEX2 was a modified version of the method described by Lecellier and Silar (1994). Plant tissues (root tips ca. 5 - 10 mm in length of 42days *in vitro* shoot cultures) were placed in a 1.5 ml Eppendorf tube with 50µl DNA extraction buffer consisting of 10mM Tris-HCl, 1mM EDTA, 100mM NaCl and 2% SDS, pH 8.0 using a sterile alcohol flamed forceps. Tubes were vortexed and the following cycle repeated four times - tubes frozen for 30s in liquid nitrogen then heated at 70°C until completely thawed (60s) and vortexed for 30s. After the cycles, the tubes were centrifuged for 10min at

Table 1. PCR reaction mixture

Components	Amount per reaction (µl)
10 x buffer	3.30
MgCl ₂ (50mM)	1.00
d(A,C,G,T)TPs (10mM)	0.50
Template DNA (plant DNA)	1.00
Enzyme (DNA polymerase)	0.25
Oligonucleotide primer	1.00
Analytical water	25.95
Total	33.00µl

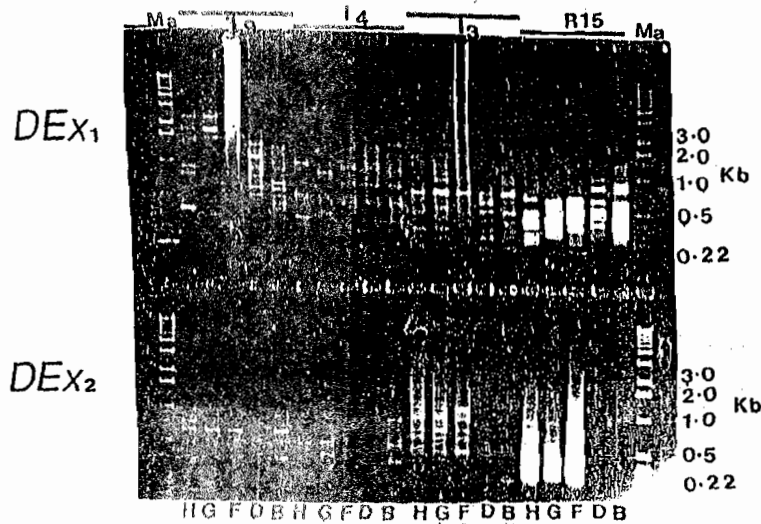


Figure 1. Polymorphic RAPD profiles of five clones of *P. esculentus* amplified by primers I₁, I₄, I₃ and R₁₅. Lanes H, G, F, D, and B represent clones VIII, VII, VI, V and IV, respectively. Ma = 1Kb ladder

13,000rpm in a microcentrifuge. The supernatant was transferred to a new tube, in which an equal volume of Phenol:Chloroform (1:500 μ l) was added, followed by centrifugation at 13,000 rpm for 5min. The upper aqueous phase of solvent was transferred to another tube. To this tube an equal volume of cold isopropanol (Propan - 2 - ol, -20°C) was added and mixed thoroughly. DNA was precipitated at -20°C for 1 - 2h. DNA was then pelleted at 13,000 rpm for 10min and dried at room temperature for 1 - 2h. The pelleted DNA was later resuspended in 200 μ l of T₁₀E₁ (10mM Tris/Hcl and 1mM EDTA, pH 8) and briefly vortexed. One μ l of the resuspended dilution was used in a 33 μ l PCR reaction (Table 1)

PCR analyses

Four arbitrary ten - Oligonucleotide (10-mers) primer sequences (1 Operon R series - R₁₅ and three Operon I series - I₃, I₄ and I₉) (Operon Technologies, USA) were used to detect polymorphism in PCR generated products derived from DNA extracted from five clones of *P. esculentus*. Primer R₁₅ was subsequently used to detect polymorphism in PCR generated products derived from DNA extracted from twelve clones of *P. esculentus*. Amplification conditions for RAPD analysis involved the use of heat - tolerant polystyrene plates (Hi-Temp 96-well

plates) on a Techne PHC - 3 dry bloc thermal cycler (Techne, Cambridge).

PCR reaction mixture

The RAPD reaction mixture contained an oligonucleotide primer, four nucleotides (dNTPs) in equal molarity, plant DNA and a thermostable DNA polymerase - *Thermus brokianus*, in a 33 μ l reaction mixture (Table 1). Reaction mixtures for the PCR were mixed in a 1.5 ml sterile Eppendorf tube. Control reaction without template DNA (replaced with sterile distilled water) was prepared for each primer. The reaction mixtures were overlaid with 50 μ l (or two drops) of light mineral oil to prevent evaporation of the sample during repeated cycles of heating and cooling. After an initial 5 min denaturation at 95°C, amplification was carried out in an automated thermalcycler programmed for 45 cycles of 1 min at 95°C, 1 min at 34°C and 2 min at 72°C of 1 min at 46°C and 2 min at 72°C. Amplified products were stored at 4°C for several days or at -20°C for much longer periods before being separated by agarose gel electrophoresis.

Electrophoresis procedure

Agarose gel for electrophoresis was prepared by weighing 3.75g agarose powder (molecular

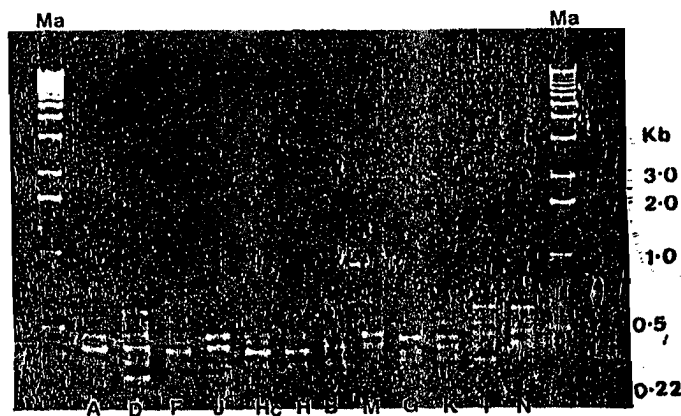


Figure 2. Polymorphic RAPD profiles of eleven *P. esculentus* amplified by R_{15} primer. Lanes A, D, F, Hc, H, B, M, G, K, I and N represent clones VTA, VTD, VTF, VTHc, VTH, VTB, LM, VTG, LK, VTI and LN, respectively. Ma = 1Kb ladder.

biology grade) (Sigma, UK) and dissolving in 250 ml 1 x TAE buffer (40mM Tris, 1mM EDTA and pH adjusted to 7.7 using glacial acetic acid). This was then boiled and allowed to cool to ambient temperature of ca. 50°C before being poured into a plastic gel casting unit. A 22-unit plastic comb was fitted to the gel casting unit prior to the gel casting to create 22 wells for the loading of amplified products. To the amplified products, 5µl loading dye consisting of 50% glycerol, 5mM EDTA and 0.25 % bromophenol was added. The amplified products and 5µl (one kilobase DNA ladder marker) were then loaded separately into the wells in the agarose gel and then electrophoresis was carried out.

Amplification products were separated by electrophoresis on a 1.5% agarose gel (20 x 20cm) in 1 x TAE buffer at 120V for 2h or 160v for 1.25h.

[TAE buffer stock solution was prepared as 50X TAE consisting of 484g Trisma base and 37.2 EDTA dissolved in 114.2 ml glacial acetic acid and the volume made up to 2l with ROW or distilled water. TAE buffer was then made by using 100 ml of the stock solution (50X TAE buffer) in 4.9l reverse osmosis water or distilled water to produce a 1l normal strength TAE buffer].

Staining

Gels were stained with ethidium bromide (0.5µg ml⁻¹ distilled water for 15 min), destained in tap water for ca 2.3 min and visualised on an UV transilluminator. The gels were then photographed

using a Polaroid MP4 camera system and film No. 665 (with negative) for interpretation and analysis.

Statistical analyses

Statistical analyses of RAPD profiles were carried out using the Multivariate Statistical Package (MVSP plus version 2.1) software (Kovach, 1993). Data manipulation involved distance and similarities with binary coefficients (the Jaccard coefficients) based on a table of frequency to matches and mismatches of the presence and absence of RAPD profiles. This was followed by a clustered analysis using the unweighted pair group method of analysis (UPGMA) based on arithmetic averages. The data was entered to a matrix as described by Kovach (1993).

Estimation of genetic distances and similarities

The genetic distances and similarities between twelve clones of *P. esculentus* evaluated in the current study were estimated using the RAPD profiles in Figure 2. Bands were scored and analysed on the basis of similarities of positions and staining intensities. Similar bands were scored as 1 when present and 0 when absent. The scores were then entered into a data matrix using the binary matrix of MVSP. Coefficients of similarity, as described by Jaccard (1908) were calculated. Similarity matrix obtained showed the similarity index of each clone

Table 2. Similarity matrix of Jaccard's coefficients showing degrees of similarities between clones of *P. esculentus* analysed

	VTA	VTB	VTD	VTF	VTG	VTH	VTHc	VTI	VTJ	LK	LM	LN
VTA	1											
VTB	0.11	1										
VTD	0.38	0.25	1									
VTF	0.57	0.29	0.43	1								
VTG	0.3	0.22	0.09	0.09	1							
VTH	0.57	0.29	0.25	0.43	0.33	1						
VTHc	0.5	0.25	0.22	0.38	0.3	0.83	1					
VTI	0.2	0.43	0.22	0.38	0.3	0.22	0.2	1				
VTJ	0.57	0.29	0.67	0.43	0.2	0.43	0.38	0.22	1			
LK	0.38	0.29	0.43	0.25	0.2	0.25	0.22	0.38	0.67	1		
LM	0.3	0.57	0.33	0.33	0.27	0.5	0.44	0.44	0.33	0.27	1	
LN	0.13	0.4	0.14	0.14	0.11	0.33	0.29	0.13	0.14	0.14	0.43	1

NB: The range of values from 0 – 1 indicates increasing similarity.

compared to all others based on the number of bands shared between (Table 2). In order to group the most similar clones together, a cluster analysis was carried out using UPGMA based on the arithmetic averages of the Jaccard similarity coefficients. A dendrogram was generated from the analysis.

RESULTS

Evaluation of the DNA extraction procedures on five clones using four primers (R₁₅, I₃, I₄ and I₉)

Four primers were tested for their abilities to detect polymorphism in PCR generated products derived from DNA extracted from five clones of *P. esculentus* (VTB, VTD, VTF, VTG and VTH). The DNA were extracted using two different procedures (DEX1 and DEX2).

From the results, DNA fragments generated by the procedure DEX1 showed polymorphism in all five clones evaluated under all primers tested, with the exception of clone VTF that showed no amplification product when primers I₃ and I₉ were used. The number of bands amplified by the other primers varied from 1 – 13 with an average of 8.6 bands per primer I₉, 9.6 per I₄, 8.8 per I₃ and 10 per R₁₅. The size of the amplified products ranged from 220bp to 3500bp (Figure 1).

DNA fragments generated by the procedure DEX2 did not show polymorphism in the five clones evaluated when primer I₉ was used. No amplification products were observed in clones VTD and VTF when primer I₄ was used. Primers I₄ and I₉ gave similar amplification products for clones VTB and VTF. Clones VTG and VTH had almost virtually similar amplified products. The same is true for clones VTF, VTG and VTH when primer I₃ was used. The only difference was the fact that clone VTF had sharper bands when compared to VTG and VTH. No amplification was observed in clone VTD, while VTB

had different banding profile. Amplification products obtained from R₁₅ were similar for clones VTF, VTG and VTH, but non observed for clones VTB and VTD (Figure 1).

In all cases the banding patterns were different from primer to primer. The number of DNA bands amplified by each primer varied from 0 – 9 with an average of 7.6 bands per primer I₉, 2.4 per primer I₄, 6.6 per primer I₃ and 8.8 per primer R₁₅.

The size of the amplified products ranged from 220bp to 2000bp as with DEX1 procedure.

Evaluation of PCR generated products derived from twelve clones of *P. esculentus* using primer R₁₅.

Further evaluation of DNA fragments generated by primer of the R series (R₁₅) following the better performance of R₁₅ in the investigation above, showed polymorphism in twelve clones of *P. esculentus*. The number of bands amplified varied from 6 – 10 with an average of 7.4 bands per clone. The band ranged from 200 – 1000bp. With the exception of clone VTH and VTHc that virtually showed similar banding patterns, almost all the amplified products seemed specific to all clones evaluated (Figure 2).

Estimation of genetic distances and similarities

The results indicated that clones VTH and VTHc were similar with a coefficient of 0.83. Clones LK and VTJ had a coefficient of 0.67 followed by VTB and LM as also VTA and VTF with a coefficient of 0.57. Coefficients of other clones are as shown in the dendrogram. The results of this evaluation further indicated that all the clones assessed using this technique were distinct from each other at much lower similarity coefficients. The clones, however, could be

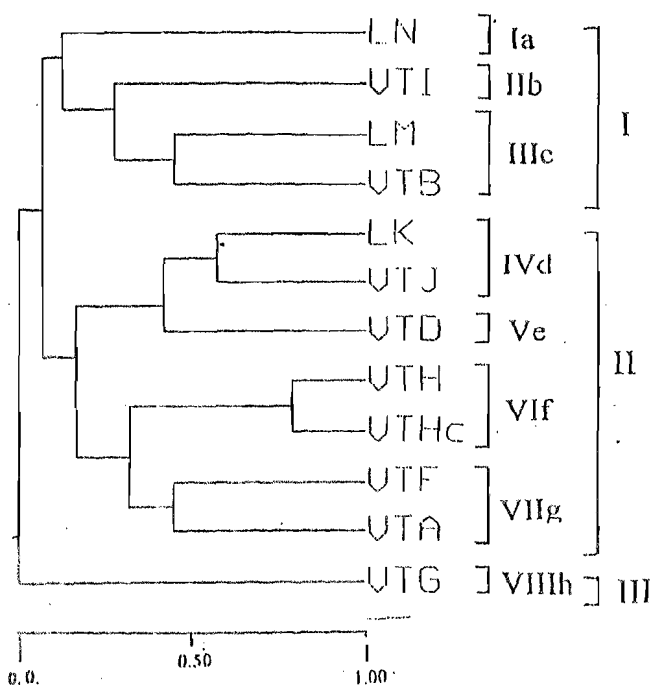


Figure 3. A dendrogram based on Jaccard's coefficients of similarity (using UPGMA) of RAPD amplified products of clones of *P. esculentus*.

grouped into one of three groups or alternatively eight sub-groups (Figure 3).

DISCUSSION AND CONCLUSION

RAPD markers based on PCR marker technique using arbitrary primers are currently being used to characterize living organisms. They have been used in the discrimination of not only plant cultivars, but also in the characterization of plant species and genera. For example, DNA profiles have been reported in rye (Iqbal and Lane - Rayburn, 1994) and *Eucalyptus* spp (Keil and Griffin, 1994). It has been used to distinguish twelve clones of *P. esculentus* (Kyesmu and Mantell, 1999). In order to develop suitable DNA fingerprinting technique for *P. esculentus* for carrying out germplasm auditing and management functions in respect of 'Rizga' germplasm collections at some stage in future, the DNA extraction procedures were evaluated. Whereas polymorphism was observed in the clones under evaluation when extraction procedure DEx1 was used, this situation was not observed in the case of DEx2. Aichitt (1993) while trying to

develop a standard protocol for the characterization of date palms, observed that systematic evaluation of DNA extraction procedures, concentration of primers and enzymes all significantly contribute in developing a standard protocol that will stand the test of time. Results obtained from the current study showed that the use of 10-mer primers differed in their capacities to generate polymorphism within the clones tested. When the primer "R₁₅" was used, all the five clones tested showed high numbers of polymorphic bands that were clonal-specific and few bands were common between clones (Figure 1). Its application in the current study to distinguish between twelve Nigerian clones of *P. esculentus* was necessary and important in view of the fact that apart from traditional morphological methods (which is already undergoing review, personal communication with A. Paton, a taxonomist with Kew Botanic Gardens) this will be the first time such a robust method of identification has been used to distinguish between clones of an indigenous African tuber crop.

Establishing relationships among these cultivars was rather difficult because the only means to characterise them so far, was the use of morphological characters, which were not easy to assess. RAPD analysis is, therefore, a useful tool in establishing the relationships among traditional cultivars found in a given locality.

The RAPD analysis results revealed high degree of genetic polymorphism with 6.4 bands per clone when primer R₁₅ was used on twelve clones. Compared with other crops, *P. esculentus* manifests high genetic variability. Previous studies on sweet potato diversity revealed 3.7, 16.7 and 4.85 bands per primer (Connolly *et al.* 1994, He *et al.* 1995 and Anselmo *et al.* 1998, respectively). Prakash and He (1996) found that USA cultivars despite their close relatedness possess sufficient variations. High polymorphism among the genotypes may be due to the different sources and nature (that is traditional, introductions and products of polycross breeding). Our results indicate a relatively wide genetic base of *P. esculentus* germplasm on the Plateau, which makes the process of selection for desirable traits difficult. This justifies the potential of DNA markers as a tool in identifying core collections to be used as parents in hybridisation blocks.

However, a large-scale RAPD analyses

should be carried out in future using other *P. esculentus* genotypes to establish available genetic markers for each of the available genotype on the Plateau. These markers can be used in future breeding programmes to identify and select high yielding or better quality genotypes of 'Rizga'

The use of isozymes as genetic markers based on the fact that these are direct products of genes which are a function of both DNA and the expression of phenotypic characters that may be subjected to banding changes, brings into focus the validity of using isozymes as genetic markers when it is considered that RAPDs unlike isozymes, allow for the detection of variations at the DNA level. Their additional advantage is that they are fast and more significantly can be detected using any tissue at any developmental stage of the plant (though in some cases chloroplast DNA may interfere with the genomic DNA analyses when samples are taken from the leaves as compared to darkened roots).

From the results obtained for the current RAPD analyses and isozymes' evaluation in our previous report (Kyesmu and Mantell, 1999), high levels of variations leading to totally different associations of clones were observed more in the former than in the later analyses. This was probably because the number of gene loci usually detected by RAPD markers in any biological object are much higher than that detected with isozymes or morphological traits (Kongkiatngam *et al.*, 1995). Estimates of genetic variations obtained with RAPD markers are usually higher because of the dominant nature and one band/one locus assumption and the fact that RAPD markers detect both coding and non-coding sequences in a genome. The non-coding sequences have been shown to be more variable than the coding sequences (Ohta, 1992). On the other hand, estimates based on isozyme polymorphism may underestimate overall levels of genetic variation because they are sampling only the coding regions that may be conserved to maintain the function of the enzymes (Gottlieb, 1982). Similarly, Liu and Furnier (1993) observed that RAPD markers gave slightly higher estimate of genetic diversity than isozymes in trembling aspen (*Populus tremuloides* Michx.) and big tooth aspen (*Populus grandidentata* Michx.) both are outcrossing tree species widely distributed in North America.

For satisfactory taxonomic practices, it is important to evaluate all the available techniques,

such as isozymes using IEF techniques and RAPD markers using the PCR techniques in order to successfully distinguish between plant groups with greater precision and confidence.

However, it is pertinent to note that the use of a RAPD marker technique for clonal identification is fast and reliable. Primers used in the screening for polymorphism in clones of *P. esculentus* in the current study showed distinct profiles for every single clone assessed with DEx1 extraction procedure. Therefore, for germplasm management of *P. esculentus*, primers that ensure polymorphic bands detection and a better DNA extraction procedure should be used for genetic fingerprinting of available clones. This, however, may vary from one management set-up to the next.

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