AVALIAÇÃO DE PROTOCOLOS PARA EXTRAÇÃO DE DNA DE AROEIRA *(Myracrodruon urundeuva)*

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RESUMO: *Myracrodruon urundeuva* (aroeira) é uma espécie arbórea de grande importância ecológica no Brasil. Entretanto, devido à intensa fragmentação e exploração predatória em sua área de ocorrência, suas populações encontram-se isoladas em pequenos fragmentos. Como forma de promover a correta conservação do germoplasma remanescente da espécie, se fazem necessárias a identificação e a caracterização de sua diversidade genética por meio de técnicas genético-moleculares, envolvendo a avaliação de um grande número de indivíduos. Nesse contexto, a extração de DNA é uma etapa básica e de suma importância para a qualidade das informações a serem geradas. Entretanto, a aroeira apresenta metabólitos secundários envolvidos na sua defesa química contra herbivoria e ação de patógenos, que podem ser fatores complicadores na extração do DNA. Assim, este trabalho teve por objetivo comparar metodologias de extração de DNA para *M. urundeuva*, empregando-se protocolos já existentes e algumas modificações. Foram testados sete diferentes protocolos e os melhores resultados foram observados quando se efetuou modificações no tampão de extração do protocolo Doyle e Doyle (1987), através do acréscimo de proteinase K (100 ug/mL), PVP-40 (4%), PVP-360 (1%) e ß-mercaptoetanol (2%). As amostras de DNA produzidas com este protocolo apresentaram bons perfis eletroforéticos, além de boa performance em reações PCR para a amplificação de marcadores moleculares microssatélites (SSR).

Palavras-chave: aroeira, microssatélites, extração de DNA, espécie arbórea tropical

EVALUATION OF PROTOCOLS FOR DNA EXTRACTION FROM AROEIRA *(Myracrodruon urundeuva)*

SUMMARY: Myracrodruon urundeuva (aroeira) is an arboreal species of great ecological importance in Brazil. However, due to the intense fragmentation and predatory exploration on its native areas, the populations are found isolated in small fragments. As a way of promoting the correct preservation of the species remaining germplasm, it is necessary to identify and characterize its genetic diversity through molecular markers, which involves the evaluation of high numbers of individuals. In this context, the DNA extraction is a basic step of great importance for the reliability and the molecular marker information. However, M. urundeuva has secondary metabolites involved in its chemical defense against herbivores and pathogens, which may hinder DNA extraction. This study describes results for comparisons on seven different methods of DNA extraction tested in M. urundeuva. The better results have been observed when modifications

have been applied to the extraction buffer from Doyle and Doyle (1987) protocol by adding proteinase K (100 ug/mL), PVP-40 (4%), PVP-360 (1%) and ß-mercaptoethanol (2%). Samples of DNA produced by this protocol showed excellent electrophoresis profile and good performance in PCR reactions for the amplification of molecular markers microsatellite (SSR).

Keywords: aroeira, tropical tree species, SSR, DNA extraction protocol

INTRODUCTION

Many authors claim difficulties in extracting good-quality DNA from different arboreal species and from other plants (Lodhi et al. 1994, Moncalvo et al., 1995, Cheng et al. 1997, Romano and Brasileiro, 1999, Mazza and Bittencourt, 2000; Vidal et al, 2003), specially due to the high level of polysaccharides and secondary metabolites, phenol oxidases action on DNA degradation. One strategy to overcome these problems is test different protocols or modification of already described extraction protocols, as to make them appropriate to the target plant species. Another possibility is the use of commercial kits for DNA extraction, which usually demand specialized instrumentation, not mentioning the high costs per samples that can be prohibitive for lab routine extractions (Goes Neto et al. 2005).

Currently, the protocol optimization is especially true for DNA extraction from native arboreal species from the Brazilian flora, considering the great number of species suitable for genetic conservation and genetic breeding studies that rely on molecular markers (Faleiro et al. 2002; Kageyama et al. 2003; Stefenon et al., 2004). Myracrodruon urundeuva, also know as aroeira, is tropical arboreal species that presents ecological and economic importance. This specie has chemical defense mechanism against herbivores and pathogenic diseases as well, which includes production of substances with insecticidal and fungicidal effects, such as tannins, and secondary metabolites originated from polyphenols (Haslam, 1989; Bernays et al., 1989; Harbone et al., 1991; Queiroz et al., 2002). All the compounds mentioned above can jeopardize extraction of plant DNA and its purity.

The objective of our study was the comparison of seven methods for DNA extraction from small amounts of M. urundeuva leaf tissue. Also, it was aiming to evaluate the quality of DNAs for PCR, obtained by the extraction method, among those tested, which resulted in the best eletrophoresis profile.

MATERIAL AND METHOD

Individuals of *M. urundeuva* were sampled from an ex situ native plants resource bank, installed as a progeny test on the Research Farm from UNESP at Ilha Solteira Campus, in Selvíria - Mato Grosso do Sul (MS), Brazil. Leaves from adult trees (20 years old) have been collected, storage in silica gel until dried, then they ground in liquid nitrogen until form a powder.

DNA extraction- Seven different extracting methods have been used as describe below. In these analyses was employed samples of leaves.

Method I (Doyle and Doyle, 1987): Powder plant tissue (150 mg) plus 750 µL extraction buffer 2X CTAB (Tris-HCl pH 8,0 100mM; NaCl 1,4M; EDTA pH 8,0 20mM; CTAB 2% and ß- mercaptoethanol 0,2%), heated up to 60 °C for 30 min., stirring occasionally. After cooling were added to each tube 450 µL CIA (chloroform / isoamyl alcohol). The samples was shaken by reversal tube, for 8 min., and centrifuged to 6000 xg, 20°C, 10 min. In supernatant collected, was added 600ul of cold isopropanol and was kept at -20 °C for 2 hours. After then, samples were centrifuged (13,000 Xg, 20 min., 20 °C) and pellets were washed for 20 min. with 1,300 µ-L wash buffer (76% ethanol / v, 10 mM ammonium acetate). After new centrifugation (13,000 Xg, 10 min., 20 °C), the "pellets" were completely dry and added up 100 µL TE [10mM Tris-HCl (pH 7.4), EDTA 1mM] plus 10 µL RNAse solution (110 ug/mL) and kept at 37 °C for 30 minutes. In

each sample was put 200 µL distilled water, 100 µL ammonium acetate 7.5 M (pH 7.7) and 1 mL of cold ethanol. The samples were leave in the refrigerator for 24 hours, and then centrifuged (13,000 Xg, 10 min., 4 °C). The pellet was dry and solubilized in 40 µL TE.

Method II (Doyle and Doyle, 1987) with modifications. In method I, described above, the following changes are maded: in extraction buffer was increased by proteinase K (100 µg/mL), PVP-40 (4%), PVP-360 (1%) and ß-mercaptoethanol (2%).

Method III (Stefenon et al., 2004): Powder plant tissue (150 mg) plus 1.5 mL extraction buffer 2X CTAB (20 mg/mL CTAB; 87 mg/mL NaCl; 20 mM EDTA; 12,1 mg/mL Tris-HCl pH 8,0; 20 mg/mL PVP; 1 mL de ß-mercaptoethanol), heated up to 60 °C for 60 min., stirring every 15 min. After reached the room temperature, it was added to each tube, 600 µL CIA (24:1). The samples was shaken by reversal tube, for 8 min. and centrifuged (13,600 Xg, 20 °C, 5 min). In supernatant collected, was added 10 µL RNAse solution (110 ug/mL) and kept in water bath (34°C, 40 min). After the addition of 1,200 µL of CIA (24:1), tubes were homogenized by 3 min. and centrifuged (13,600 Xg for 5 min.) at room temperature. The supernatant was transferred to another tube, was added 100 uL of CTAB solution 10% [100 mg/mL CTAB, 81.2 mg/mL NaCl], and after stirring, was added 600 uL CIA, reversing itself by to 3 min. The tubes were then centrifuged (13,600 Xg, 5 min. at room temperature). The supernatant was transferred again to another tube adding 2/3 the volume of isopropanol (-20 °C) and incubated at -20 °C for 30 min. The samples was centrifuged (4,000 xg, 5 min., room temperature). The pellets were washed with 1 mL of ethanol 95%, and kept at -20 °C for 10 min. The tubes were centrifuged (5,100 Xg, 5 min., room temperature), supernatant was discarded and after pellets were dried, they were solubilized in 40 µ L TE.

Method IV (Colpaert et al., 2004):Tubes containing powder plant tissue (150 mg) plus 1.5 mL extraction buffer 2X CTAB (Tris-HCl pH 8,0/ 100mM; NaCl 1,4M; EDTA pH 8,0/ 20mM; CTAB 2%, ß- mercaptoethanol 2%; PVP (40) 2%; Proteinase K 10mg/ml; SDS 10%), was mixed in the vortex and heated up 65 ºC for 60 min., stirring occasionally. Then the samples reaching the room temperature, was added 6 ?L RNAse (10mg/ml) and kept in water-bath (10 min, 37 ºC). After the addition of 600 µL CIA (24:1), the samples was shaken by reversal tube by 10 times and centrifuged (13,000 xg, room temperature, 10 min). The upper phase was collected and transferred to another tube plus 140µl CTAB 10% and 280 µl NaCl 5M. Twice added 600 µL CIA, shook up by inversion (10 times), centrifuged (13,000 xg, room temperature, 10 min) and collected the upper phase. Added 600ul cold isopropanol, kept at -20ºC for 1 h, then it was centrifuged (13,000 xg, room temperature, 20 min). The pellet was solubilized with 200µl sodium acetate 0.1M and 20 µL ethanol 96%. The tubes were placed on ice for 10 minutes, and then centrifuged (13,000 xg, 5 min., room temperature). The supernatant was collected and transferred to another tube containing sodium acetate 0.1M, 20 µL ethanol 96% and kept in ice (10 min). After centrifuged (13,000 xg, room temperature, 5 min), the upper phase was collected and added sodium acetate 3M, 440 µL ethanol 96% and kept at -20 °C (overnigth). Again it was centrifuged (13,000 xg, room temperature, 20 min), the pellet was dried, and solubilized in 40µL destilled and autoclaved water.

Method V (MasterPure™ Plant Leaf DNA Purification Kit - Epicentre, Madison, USA): Powder plant tissue (100 mg) was put in 1.5 mL microcentrifuge tube containing 300uL Plant DNA Extraction Solution. The sample was heated for 30 minutes at 70°C. Then it was chilled on ice and centrifuged (13,000 xg, room temperature, 10 min) to pellet cellular debris. The supernatant was transferred to a fresh tube and repeat the centrifugation step. Again the supernatant was transferred to a fresh tube and the DNA was precipitated with one volume isopropanol cold, and centrifuged (13,000 xg, room temperature, 5 min). The pellet was washed with 70% ethanol, and after dried, the pellet was resuspended in 25 uL TE Buffer.

Method VI. (Lodhi et al., 1994): Powder

plant tissue (500mg) plus 5 mL extraction buffer (20mM EDTA, 100mM Tris HCl, pH 8,0, 1,4M NaCl, 2% CTAB, 0,2% ß-mercaptoethanol, 50 mg PVP) was heated for 25 minutes at 60°C. After the samples reaching the room temperature, was added 6 mL de CIA (24:1), shaked by reversal tube, for 25 times, and centrifuged (6,000 rpm, room temperature, 15 min). The supernatant was transferred to a fresh tube with 6mL CIA (24:1), shaked by reversal tube, for 25 times, and again centrifuged with the same conditions. The supernatant was collected and added 2mL NaCl 5 M, 8mL ethanol 95% cold, kept at 10°C for 1 h. After centrifugation (3,000 rpm, 3 min. and 5,000 rpm, 3 min), the pellet was washed with 76% cold ethanol, oven-dried (37°C, 30 min) and resuspended for 1h in 125 uL TE buffer. Finally was added 2uL RNAse A (10 mg/mL) and kept at 37ºC for 15 min.

Method VII (Vidal et al., 2003): Powder plant tissue (500mg) plus 1.1 mL extraction buffer (4M urea, 50mM EDTA and 250mM Tris HCl, pH 8,0, 250mM NaCl, 2% SDS, 5% de b-mercaptoethanol), 30 uL de proteinase K (10 mg/mL) was heated up to 50 ºC for 60 min., stirring every 15 min. The samples were centrifuged (2000 Xg, 10 min), the supernatant was colleted and added 1 v potassium acetate (5M) with vigorous shaker. The samples were putted on ice bath (30 min) and centrifuged (9,000 Xg, room temperature, 5 min). In collected supernatant was added 1v of CIA (24:1) and centrifuged (11,000 Xg, 12 min) . This step was carried twice. In the collected supernatant was added 1v of cold isopropanol, and the samples were kept at -20°C (overnight). After centrifugation (11,000 xg, 10 min), the pellet was washed with 70% cold ethanol, dried and resuspended in 125 uL TE buffer.

DNA electrophoresis conditions

Electrophoresis profile was employed for the evaluations of quality and integrity of the genomic DNAs extracted. Thus, samples (10uL DNA) were submitted to horizontal eletrectrophoresis in 0.8% agarose gel in 1X TBE buffer (Tris, 28 mM; boric acid, 88 mM; EDTA, 7 mM; pH 8.3) containing ethidium bromide (5 µg/mL), at constant tension 80V for about 1h 30 min.

DNA quantification and PCR (polymerase chain reaction)

In these analyses, was employed only DNA genomic extracted with the method considered, in this investigation, more appropriated, in terms of practicality and shows good profile eletrophoresis. The DNA quantification was done in 440 samples, by spectrophotometry, taking into account that one unit of absorbance at 260 nm wavelength is equivalent to 50 µg of double stranded DNA (Sambrook et al., 1989). Measures in 230/280 nm and 260/280 nm were taken to verify the sample purity.

DNA samples were randomly selected and employed in SSR-PCR reactions, using primers (Auru: B209, J185, E062, C072, D167, D094) and conditions of amplification proposed by Caetano et al. (2005). For amplification of alleles used to thermocycler Eppendorf MasterCycle with following program: one cycle of 94 °C for 3 min., 35 cycles of 94 °C for 30 sec., temperature melting of 30 sec., 72 °C by 30 sec., and finally one cycle of 72 °C for 5 min.

RESULTS, DISCUSSION AND CONCLUSIONS

The Method I (from Doyle and Doyle, 1987) was not effective for extracting DNA from M. urundeuva samples that were used in this study, since no DNA electrophoresis profile was observed, indicating total absence of DNA (Figure 1). Some authors pointed this method (I) as proper for DNA extraction from M. urundeuva (Freitas et al., 2005; Freitas et al., 2006; Reis & Grattapaglia, 2004). An important factor that must be considered concerns the age of the plant material used in our study, which was distinct from others studies. M. urundeuva is a deciduous specie, and in this study, we have leaves collected in a period

near the fall of them, from adult plants (20 years old). In contrast, Freitas et al. (2005) and Freitas et al. (2006) have used leaves in the beginning of shooting, and Reis & Grattapaglia (2004) have used young leaves from greenhouse plants (from 3- to 4-month-old), thus much younger than the ones we used. Faleiro et al. (2002) consider the type and the age of the plant tissues are important factors that influence the quality of the DNA samples. Thus, state of maturation of both the leaves as the plant has important implications in metabolites found in the leaves. Some researchers consider that, the polyphenolic compounds - often involved in the defense against herbivores, are either absent or found in low concentrations in younger leaves (Mitton et al. 1979). Probably these compounds were present in highest concentration in the leaves used in this study, which may explain the fact the method I has failed with the leaf samples from this study.

Method II yielded good results (Figure 2), probably due to a synergism of the modifications implemented on Doyle & Doyle (1987) protocol. The increase in ß-mercaptoethanol concentration combined with the presence of two antioxidants (PVP-40 and PVP-360) must have lead to strong anti-oxidizing effect on secondary metabolites presents in leaves. The modification also included the addition of proteinase K, which must have had a proteolytic action on the proteins that were hindering the DNA availability in the samples.

Method III was considered by Stefenon et al. (2004) as appropriate protocol for protecting plant DNA from oxidizing, since it uses the antioxidant PVP-40. Furthermore, this protocol is postulated to intensify the soluble effect on cell membranes as it uses CTAB. Another protocol considered similarly effect is Method IV, as it employs antioxidants too. It uses two anti-oxidizing agents (PVP-40 and ß-mercaptoethanol) and it also a protease (proteinase K), which removes proteins from the samples. This method was reported as an efficient DNA extraction protocol for several plant species, such as *Brosimum guianense, Carapa guianensis, Ceiba pentandra, Eschweilera costaricensis, Goethalsia meiantha, Laetia procera, Lecythis ampla, Maranthes panamensis, Swietenia macrophylla, Tetragastris panamensis and Vochysia ferruginea* (Colpaert et al., 2005). Nevertheless, it was not possible to extracted DNA from *M. urundeuva* samples, in present investigation, with neither one of these two methods. No electrophoresis profile could be observed for aroeira DNA samples extracted with either Method III or Method IV, similar to what was presented in Figure 1. Even though the anti-oxidizing agents PVP and ß-mercaptoethanol have been used, some samples showed darkened DNA pellets. These dark pellets are probably formed due to the action of phenolic compounds present in the plant samples and which irreversibly oxidize DNA, making it inaccessible and non-useful for further genetic analyses with molecular markers (Romano & Brasileiro, 1999). A similar condition of dark DNA pellets was found with Methods VI. High levels of tannins and other polyphenolic compounds found in leaves can cause cell oxidation during DNA extractions, (Coley, 1983; Turner, 1995; Coley & Barone, 1996; Stefenon et al., 2004).

Method V consisted of using a commercial kit which, according to the producer is indicated even for DNA extraction from problematic leaf tissues, containing polyphenolic compounds, tannins and polysaccharides (Epicentre, Wisconsin, USA). This method was satisfactory for aroeira's DNA extraction, although intensity of the genomic DNA bands was weak in the electrophoresis profile (Figure 3), indicating low concentration of DNA. Smear was observed in some samples, indicating that the integrity of the same could be compromised. Caetano et al. (2005, 2008) have also used commercial kits for DNA extraction in their study on population diversity of *M. urundeuva* from South American populations. Due to the high cost of these kits, their use can become impracticable in population diversity, conservation and plant breeding studies that involve a great number of samples. If costs are not a concern, this

method could be the best choice, as it is very practical and efficient (Goes Neto et al. 2005).

Methods VI and VII were not effective for extracting DNA from the aroeira samples that were used in this study, since no DNA electrophoresis profile was observed, indicating total absence of DNA, similar it was present in figure 1. It was expected a better performance of the method VII, since it has the advantage simultaneous use of two detergents, SDS (sodium dodecyl sulfate) and CTAB (cetyltrimethylammonium bromide), which act making the membranes soluble and eliminating polysaccharides from samples (Romano and Brasileiro, 1999).

The fact of the methods I, III, IV, VI and VII were not effective in extracting DNA of samples in this study, may have been caused by the presence of polyphenolic compounds in leaf tissue, as already reported by Queiroz et al. (2002). These compounds may have damaged the DNA molecule. Other possibility is that these DNA extractions procedures resulted in a molecular complex between DNA and other macromolecules, resulting on DNA shearing during purification process. One of the difficulties for DNA extractions from plants tissue is the co-isolation of polysaccharides and other secondary metabolites. When combined with nucleic acids, these compounds make the samples extremely viscous and hinder their migration on gel electrophoresis runs, justifying no DNA electrophoresis profile (Molinari & Crochemore, 2001, Romano & Brasileiro, 1999).

So, only the methods II and V were efficient in the extraction of DNA, but method II showed the best results in terms of electrophoretic profiles for the samples used in this study. However, a good profile eletrophoresis is not enough to ensure that the DNAs are appropriate to the PCR (polymerase chain reaction) technique, the most used in the evaluation of molecular markers when objective genetic breeding and / or investigations of genetic diversity. It is known that compounds co-isolated with the DNA can inhibit the activity of Taq DNA polymerase enzyme on the amplification of molecular markers by polymerase chain reaction (PCR) (Vidal et al., 2002, Colpaert et al. 2005).

Thus, DNA samples extracted by the method II, was carried out in PCR aiming to verify if their quality were adequate for employing in molecular markers analysis. All the six primers pairs resulted in positively amplified, that show the performance of the DNA samples extracted via Method II was suitable for PCRs analyses (Figure 4).

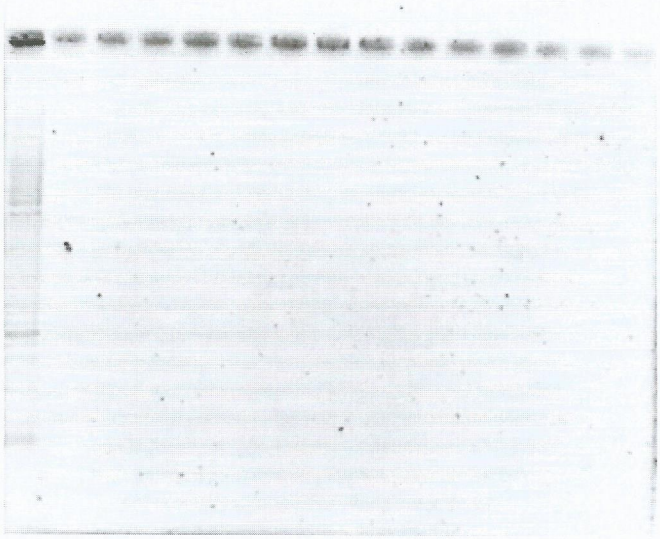

Figure 1. Agarose gel electrophoresis profiles showing the absence genomic DNA from aroeira (Myracrodruon urundeuva) when was employed Method I (Doyle & Doyle, 1987), Method III (Stefenon et al., 2004) , Method IV (Colpaert et al., 2004), Method VI (Lodhi et al., 1994), and Method VII (Vidal et al., 2003). M- molecular leader.

Figure 2. Agarose gel electrophoresis profiles showing bands of genomic DNA from aroeira (*Myracrodruon urundeuva*) when was employed Method II (modified DOYLE and DOYLE (1987) protocol).

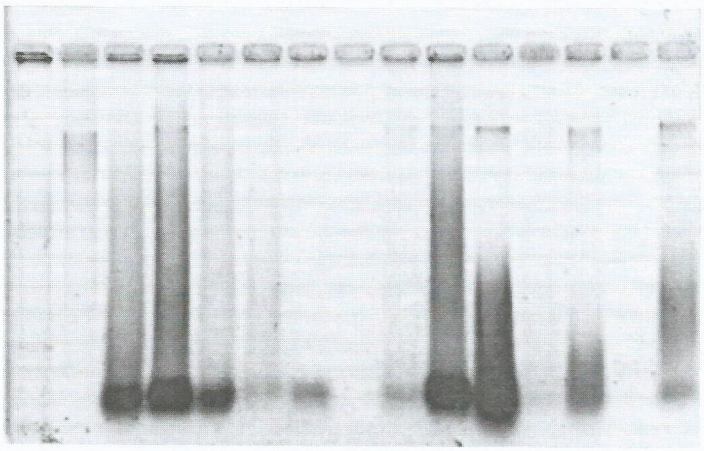

Figure 3. Agarose gel electrophoresis profiles showing bands of genomic DNA (narrow arrow) from aroeira (*Myracrodruon urundeuva)* extracted via Method V (MasterPure™ Plant Leaf DNA Purification Kit). Note occurrence smear (large arrow)

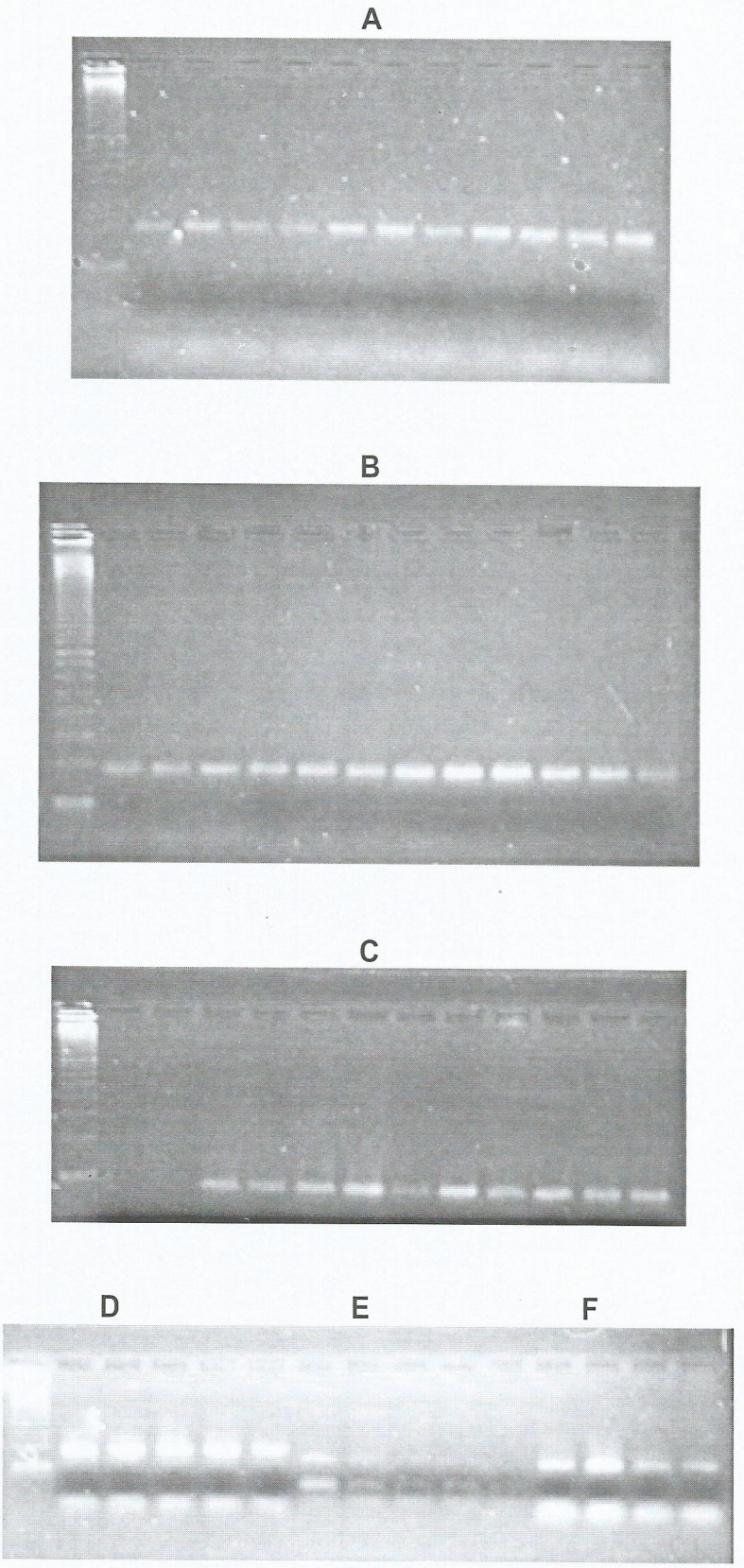

Figure 4. Agarose gel electrophoresis profiles of SSR loci amplified from Myracrodruon urundeuva genomic DNA extracted via Method II [modified Doyle and Doyle (1987) protocol]. SSR loci: A, Auru B209, B, Auru J185; C, Auru E062; D, Auru C072, Auru D167 and Auru D094)

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