Revista Ciência Agronômica, v. 56, e202392001, 2025
Centro de Ciências Agrárias - Universidade Federal do Ceará, Fortaleza, CE
www.ccarevista.ufc.br

Scientific Article
ISSN 1806-6690

Non-destructive method for extracting DNA from cashew seeds[1]

Érika Beatriz de Lima Castro[2], Men de Sá Moreira de Souza Filho[3], Patricia do Nascimento Bordallo[4]*

ABSTRACT - The characterisation and evaluation of accessions are important steps in both the management of a germplasm collection and in a plant genetic improvement program. Carrying out characterisation and evaluation, whether morphological or molecular, requires observing the overall features of plants in the field, or collecting leaves for DNA extraction, which is time consuming and costly; whereas DNA extracted directly from the seeds allows access to the genome without the need for planting the seeds, which speeds up the selection of genotypes of interest and information on genetic variability. The aim was to develop a non-destructive method for extracting DNA from cashew seeds. The seeds were mechanically perforated using a mini-grinder, and a sample of the endosperm was removed. Various protocols and commercial kits for DNA extraction were tested, as well as materials to seal the opening made in the seed. It is possible to extract cashew tree DNA from the endosperm, preferably using commercial kits with cementitious adhesive as the sealing material, offering a germination percentage of 60% of the perforated seeds.

Key words: *Anacardium occidentale* L. Anacardiaceae. Molecular markers. Genetic resources.

DOI: 10.5935/1806-6690.20250008
Editor-in-Chief: Prof. Salvador Barros Torres - sbtorres@ufersa.edu.br
*Author for correspondence
Received for publication 22/08/2023; approved on 06/03/2024
[1]This study was funded by the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) as part of Project 20.19.01.015.00.00, and is registered in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (Sisgen) under reference AF2BAA5
[2]Universidade Federal do Ceará, Fortaleza-CE, Brazil, erika-beatriz@hotmail.com (ORCID ID 0000-0002-1396-9967)
[3]Embrapa Agroindústria Tropical, Fortaleza-CE, Brazil, men.souza@embrapa.br (ORCID ID 0000-0003-4404-2360)
[4]Embrapa Agroindústria Tropical, Fortaleza-CE, Brazil, patricia.bordallo@embrapa.br (ORCID ID 0000-0003-4032-3106)

INTRODUCTION

The cashew (*Anacardium occidentale* L.) is a native species that is highly valued by the population due to its many uses and benefits, especially as food and for medicinal purposes (Novaes; Novaes, 2021). It is also of socioeconomic importance as it is a source of employment and income for families, particularly during the dry season (Brainer; Vidal, 2021). The northeast of Brazil stands out as the largest producer, with 121.2 thousand tons, while the main producer in the area is the state of Ceará, with 68.1 thousand tons (IBGE, 2023).

The characterisation and evaluation of different accessions in Active Germplasm Banks (AGB) are essential steps in understanding and accessing the genetic variability of the conserved materials (Oliveira *et al*., 2020), with this knowledge possibly allowing the accessions to be used in genetic improvement programs (Oliveira *et al*., 2020). Conserving the genetic diversity of agricultural crops is important in order to provide a genetic base (alleles) for possible adaptations that may be needed in the future (Burle, 2019).

The characterisation of conserved accessions is carried out through the use of descriptors, which are based on the morphological, agronomic, physiological and molecular characteristics of the plant, among others (Faleiro *et al*., 2020). However, using all of the descriptors requires a lot of time and manpower (Oliveira *et al*., 2020). Genetic variability can be estimated using PCR-based molecular markers (Paiva *et al*., 2019), such as SNP, SSR, AFLP, RFLP, RAPD and ISSR (Dwiningsih; Rahmaningsih; Alkahtani, 2020). Nevertheless, to carry out molecular characterisation, the DNA must first be extracted from the plant material.

Extraction is generally carried out on leaf tissue. However, when the seeds are used, molecular characterisation can be achieved regardless of the stage of the plant and before sowing, saving time and space (Von Post *et al*., 2003). This makes it possible to speed up the genetic analysis of genotypes, and characterise and eliminate seeds that have undesirable genetic traits before they are planted in the field (Ma *et al*., 2019). However, in order to sow previously evaluated and selected seeds, the genetic material (DNA) must be extracted in a non-destructive way (Zheng *et al*., 2015).

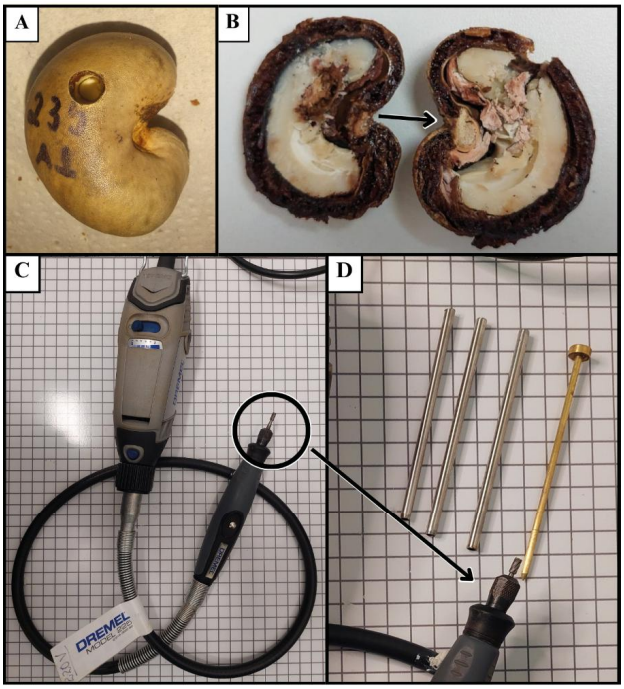
Currently, the cashew is mostly conserved by planting in the field and keeping seeds in cold storage. (Castro, 2020). However, some accessions are only conserved as seeds, as they are collected in geographically distant regions, and planting them in the field for study would take time. Developing or adapting methods for extracting DNA directly from the seed is therefore essential. Studies carried out on the cashew, seeking to facilitate DNA extraction, can simplify and optimise many of the steps, allowing results to be obtained more quickly. As such, the aim of this study was to develop a non-destructive method for extracting DNA from cashew seeds.

MATERIAL AND METHODS

Collecting the material

Seeds of the CCP 06 clone (2022 harvest) were used, collected in the experimental area of Pacajus, Ceará (04°11’07’’ S and 38°30’07’’ W), and selected for their high germination capacity. The seeds were drilled in the most distal part relative to the embryo (Figure 1) with the aid of a mini-grinder (Dremel, USA) using a No 194 countersink drill (1/8 inch) to reach the endosperm. A small sample of the endosperm (≥ 20 mg) was collected with a previously autoclaved steel tube, 3 mm in diameter, and immediately placed in a microtube with the aid of a steel pin (Figure 1). A new autoclaved tube was used for each sample. The steel tubes were used to remove the required amount of endosperm more easily and without loss, since its diameter is similar to that of the drill.

Figure 1 - Perfurated seeds of the CCP 06 clone of *Anacardium occidentale* L. and the equipment used



(A) External view; (B) Internal view (The black arrow indicates the position of the embryo); (C) mini-grinder; (D) steel tubes used to remove the endosperm

Selecting the sealing material and preliminary germination test

Four materials were tested to seal the opening made in the nut to remove the endosperm and ensure the seed remained viable, allowing it to be sown in the future. The materials were applied at room temperature using a sufficient amount to close the opening. In the preliminary tests, four materials with sealing properties were applied to the open region immediately after removing the endosperm and coat: 1) chitosan-based gel, 2) silicone, 3) cementitious adhesive and 4) plaster, the last three being commercial products.

The chitosan-based sealant was prepared as follows: 1) a solution was prepared using 10 mL of PA acetic acid (purity 99.5%) and 90 mL of distilled water; 2) 1 mL of SPAN 80 (surfactant) and 10 mL of glycerol were added; 3) the mixture was heated to 80 ºC while mechanically stirred at 300 rpm, and 5 grams of chitosan was slowly added until completely dispersed and a gel was formed; 4) the gel was cooled to 65 ºC and then, while mechanically stirred at 300 rpm, 12 grams of commercial bovine gelatin was added until completely dispersed and incorporated/ solubilised in the gel; 5) the gel was cooled to room temperature (25 ºC – 28 ºC) and was applied to the holes in the seeds using a 3-mL syringe. The cementitious adhesive and plaster were prepared as per the manufacturers' instructions and inserted using a 3 mL syringe. The silicone was applied to the holes using the applicator nozzle of the tube.

Once the sealants had dried as suggested by the manufacturers, the seeds were sown in trays containing commercial substrate (Germina Plant®). Ten perforated and unperforated (control) seeds of the CCP 06 clone were used in the preliminary tests under shaded conditions (24 °C – 31 °C) and evaluated once a week for 30 days, the criterion being the emergence of the first leaf. To select the sealant, a comparison was made between the sealing materials, choosing the material that did not drastically reduce seedling emergence.

Definitive germination test

After selecting the sealant with the best potential to seal the seeds in the preliminary tests, a germination test was carried out with two treatments: perforated and sealed seeds, and unperforated seeds (control), using 100 seeds per treatment. A completely randomised design (CRD) was used. The seeds from each treatment were sown in small tubes (288 mL) containing commercial substrate and evaluated once a week for one month to quantify emergence. The germination experiments were conducted in the seedling production nursery of Embrapa Agroindústria Tropical, in Pacajus, Ceará. The data were subjected to analysis of variance by F-test, using the Genes software (Cruz, 2016).

Extracting DNA from seeds of the CCP 06 clone

Five protocols for extracting DNA were tested: Zheng *et al*. (2015), CTAB-PVP (Costa *et al*., 2015), WIZARD PVP (Costa *et al*., 2015), Ahmad, Ferguson and Southwick (2003) and Doyle and Doyle (1990), and three commercial kits, the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), EasyPure Plant Genomic DNA Kit (TransGen, Beijing, China) and Quick-DNA Plant/Seed Miniprep Kit (Zymo Research Corp., Orange, CA). After extraction, DNA from each of the samples was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and the total DNA was visualised on 0.8% agarose gel. Methods were selected that enabled DNA of sufficient quality to be extracted for future use.

PCR for checking the viability of the extracted DNA

Verification of the viability of the DNA extracted from the CCP 06 clone using each protocol or commercial kit was carried out by PCR reaction using four ISSR primers (Table 1).

The concentrations adopted for the final volume of 20 µL consisted of: 1 X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 1 U Taq DNA polymerase, 0.8 µM primer, 20 ng DNA and ultrapure water to make up the final volume. For the PCR reactions, the samples were amplified using a thermocycler (Veriti 96-Well Thermal Cycler, Applied Biosystems) as follows: (I) initial denaturation of the DNA at 94 °C for five minutes; (II) 40 cycles at 94 °C for one minute, annealing for 40 seconds at specific temperatures for each primer (Table 1), extension for two minutes at 72 °C; (III) final extension of the DNA for five minutes at 72 ºC.

Validating the protocol in seeds of the Cerrado cashew

After defining the protocol that gave the best results for extracting the DNA, the methodology was tested on five seeds from the BGC 676 accession (2019 harvest) collected in the Brazilian Cerrado in the district of Campos Belos, Goiás (13°00'24" S and 46°36'11" W, altitude 633 m), to check that it worked with other accessions. The total DNA

Table 1 - ISSR primers used in DNA amplifications of the CCP 06 clone of *Anacardium occidentale* L.

ISSR Primers	Complete sequence (5'-3')	Ta (°C)
I826	$(AC)_8C$	47.1
I834	$(AG)_8YT$	50.4
I840	$(GA)_8YT$	49.2
I846	$(CA)_8RT$	50.0

Ta = annealing temperature. Y= C ou T; R = A ou G

was quantified using the NanoDrop 2000 (Thermo Scientific) and visualised on 0.8% agarose gel. PCR was carried out using an ISSR-type marker to validate the methodology using the I848 primer (complete sequence 5'-3': (CA)8RG) under the PCR conditions described above.

RESULTS AND DISCUSSION

Comparing the sealants and preliminary germination test

After carrying out the preliminary tests, any seeds that did not germinate were opened for internal analysis, which showed excessive water absorption, causing the kernel to rot. The sealants that proved to be hygroscopic, such as chitosan-based gel and silicone, were therefore discarded. Between the gypsum and the cementitious adhesive, the latter resulted in the least loss of germination potential, with 44% and 67%, respectively.

Definitive germination test

Sixty per cent of the perforated seeds germinated, compared to 81% of the seeds that were unperforated (control). There were no inconsistent visual or structural differences between the plants produced from the perforated or unperforated seeds. The results, however, were found to be significant (Table 2), indicating that statistically there was a difference, despite the germination percentage being greater than 50%. This shows that further studies are needed to develop adaptations and improvements to increase the germination percentage to levels close to those of unperforated seeds.

Preparing the sample for DNA extraction

Some adaptations were made to the methodologies in order to improve the results, for example, storing the endosperm samples in an ultra-low-temperature freezer at -80 °C to avoid oxidation and maintain integrity until the start of DNA extraction. After various tests, it was decided not to use liquid nitrogen on the samples, as it made maceration difficult due to freezing and the consequent hardening of the sample inside the microtube. The lysis solutions were added directly to the microtube, and the sample was macerated using an autoclaved plastic pestle for all the protocols and kits under evaluation. This avoided the possible contamination or loss of material during transfer from the crucible to the microtube in the case of maceration using liquid nitrogen. It is recommended that the operator of the mini-grinder be trained beforehand to avoid removing too much endosperm or damaging the seed.

Table 2 - Summary of the analysis of variance: source of variation (SV) and coefficient of variation (CV) for the germination test. Source: the author (2023)

SV	Germination (%)
Treatment	57.8**
CV (%)	19.2

** Significant at 1% probability by F-test

Extracting DNA from the CCP 06 clone

The bands were more defined for the DNA of the CCP 06 clone extracted with the kits, despite showing low concentrations when quantified, indicating better purification (Figure 2 and Table 3).

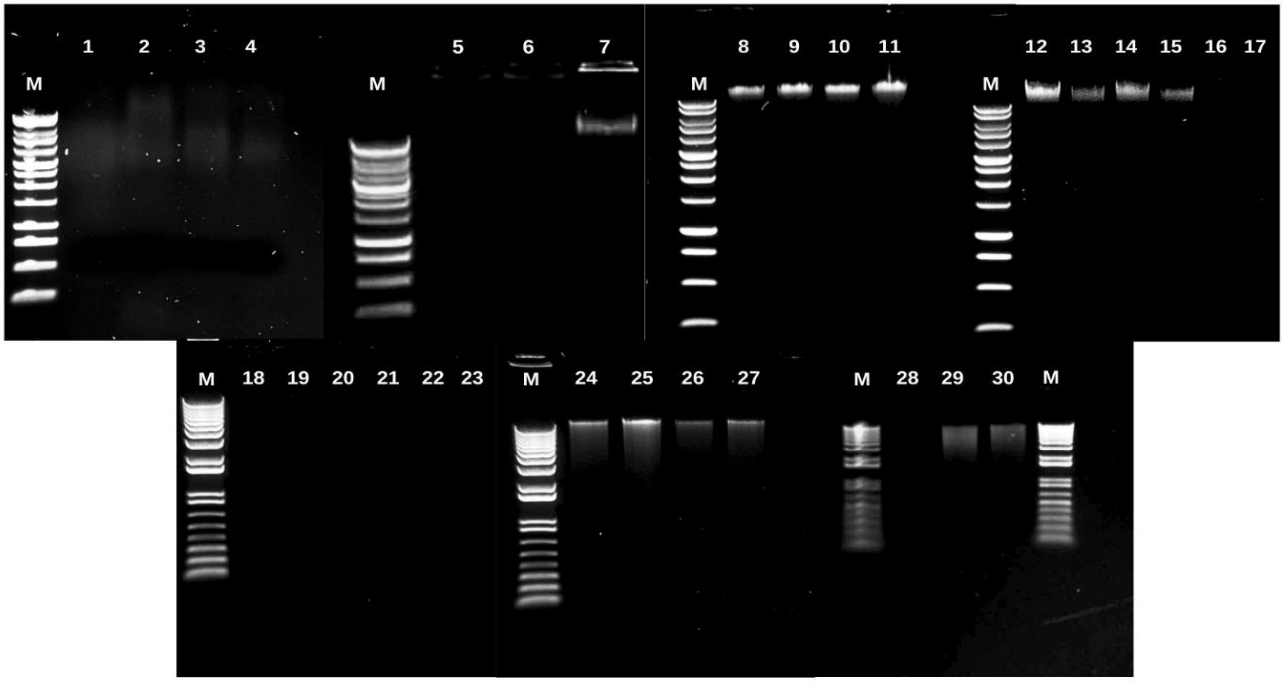
Variation in DNA quality has also been seen in other studies using extraction directly from seeds, but this does not interfere in analyses using molecular markers. Extractions carried out on barley seeds provided DNA of medium quality in low concentrations, but which could be used directly in routine laboratory analyses (Von Post *et al*., 2003). Furthermore, DNA extracted from the cotyledon tissue of cotton seeds also showed little variation in yield, but did not prevent the DNA being used for molecular analysis (Zheng *et al*., 2015). The authors also reported that using these methods for extracting from seeds, it is possible to optimise the time, operation and extraction of thousands of samples a day.

PCR for checking the viability of the DNA extracted from the CCP 06 clone

In the tests using ISSR primers carried out on each of the samples, amplification of the DNA extracted using the kits gave the best results (Figure 3). However, based on the 260/280 and 260/230 ratios and the agarose gel, the quality of the DNA was not very good. This may be due to the presence of substances that are characteristic of some plants of family Anacardiaceae and that are partly toxic, such as flavonoids, particularly biflavonoids, and phenolic lipids (Correia; David; David, 2006). These compounds can interfere with molecular analyses using PCR-based markers (Silva, 2010), and are another factor that can influence the quality of the result.

The protocol developed by Doyle and Doyle (1990) and the commercial Wizard Genomic DNA Purification Kit (Promega) and EasyPure Plant Genomic DNA Kit (TransGen) resulted in a sufficient quantity of DNA of acceptable quality. These protocols can therefore be considered the most suitable for obtaining total DNA of a quality that will allow future studies, such as analyses using molecular markers. There is a tendency to choose commercial kits, as they are faster and do not require the use of toxic reagents, such as beta-mercaptoethanol and chloroform. In addition, the cost/benefit ratio should also be considered when choosing which extraction kit to use.

Figure 2 - Agarose gel showing the results of total DNA extractions using the protocols and kits tested with the CCP 06 clone of *Anacardium occidentale* L.



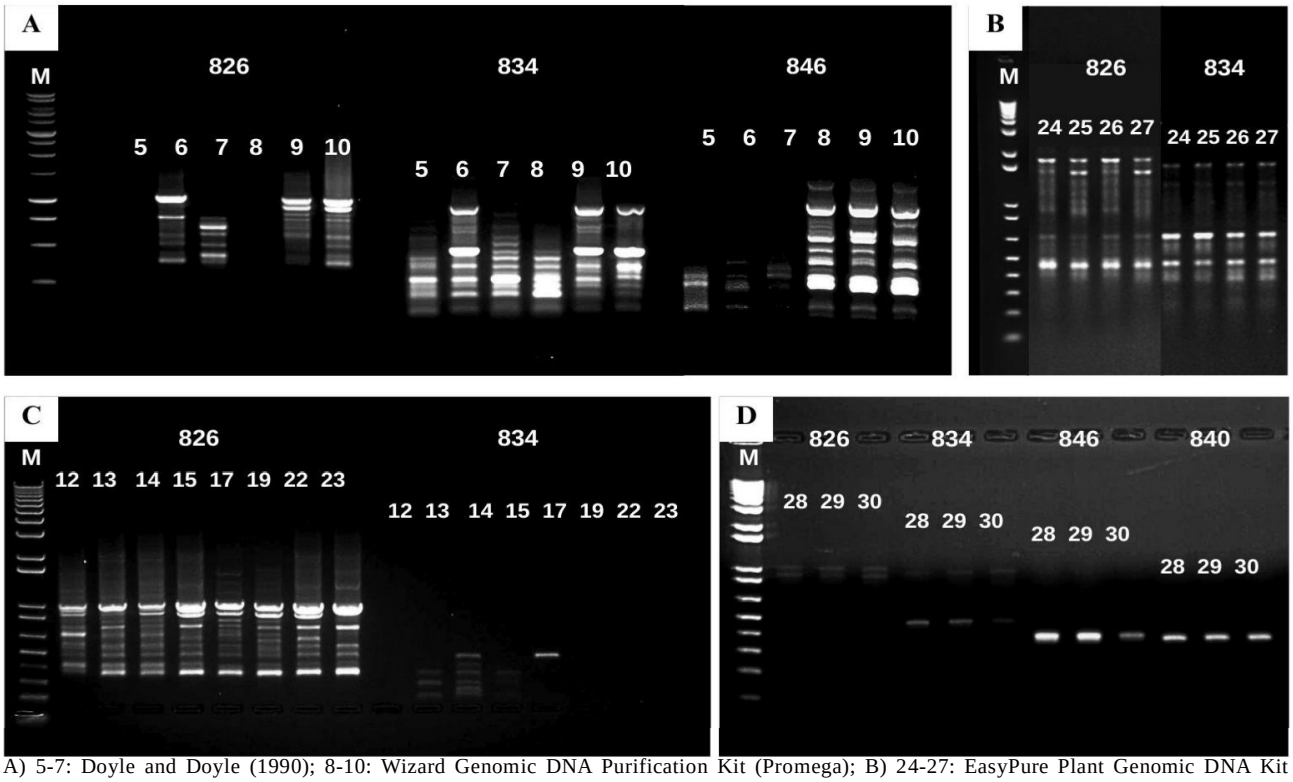
1-4: Zheng *et al.* (2015); 5-7: Doyle and Doyle (1990); 8-11: Wizard Genomic DNA Purification Kit (Promega); 12-15: Wizard PVP - Costa *et al.* (2015); 16-19: Ahmad, Ferguson and Southwick (2003); 20-23: CTAB-PVP – Costa *et al.* (2015); 24-27: EasyPure Plant Genomic DNA Kit (TransGen); 28-30: Quick-DNA Plant/Seed Miniprep Kit (Zymo). The numbers in each gel column identify the seeds that were used. M: 1Kb molecular weight marker (Promega)

Table 3 - Quantification and evaluation of the quality of the DNA from the CCP 06 clone of *Anacardium occidentale* L. using the NanoDrop 2000 (Thermo Scientific)

ID	Concentration (ng/µL)	260/280	260/230	ID	Concentration (ng/µL)	260/280	260/230
01	217	0.92	0.31	16	19.6	0.95	0.43
02	336.4	1.33	0.44	17	19.9	1.01	0.22
03	310.7	1.46	0.49	18	43.2	0.91	1.07
04	151.4	1.51	0.49	19	21.6	1.2	0.35
05	52.5	1.73	0.69	20	22.3	2.16	2.33
06	301.2	1.86	1.07	21	6.9	2.88	-14.1
07	1074.8	1.55	0.54	22	21.4	2.01	1.89
08	722.4	1.38	0.54	23	10.0	1.77	2.83
09	209.7	1.75	0.95	24	7.4	2.86	0.2
10	83.2	1.2	0.44	25	2.5	3.65	1.64
11	392.3	0.9	0.21	26	49.1	1.57	0.77
12	190.5	1	0.27	27	9.0	1.55	1.24
13	50.3	1.25	0.38	28	6.6	-3.74	-0.6
14	75.5	1.08	0.28	29	7	-6.92	0.25
15	158	1.62	0.68	30	6.4	-3.14	-2.68

1-4: Zheng *et al.* (2015); 5-7: Doyle and Doyle (1990); 8-11: Wizard Genomic DNA Purification Kit (Promega); 12-15: Wizard PVP - Costa *et al.* (2015); 16-19: Ahmad, Ferguson and Southwick (2003); 20-23: CTAB-PVP – Costa *et al.* (2015); 24-27: EasyPure Plant Genomic DNA Kit (TransGen); 28-30: Quick-DNA Plant/Seed Miniprep Kit (Zymo)

Figure 3 - Results of the amplifications of DNA from the CCP 06 clone of *Anacardium occidentale* L. using ISSR primers, for the different extraction protocols under test



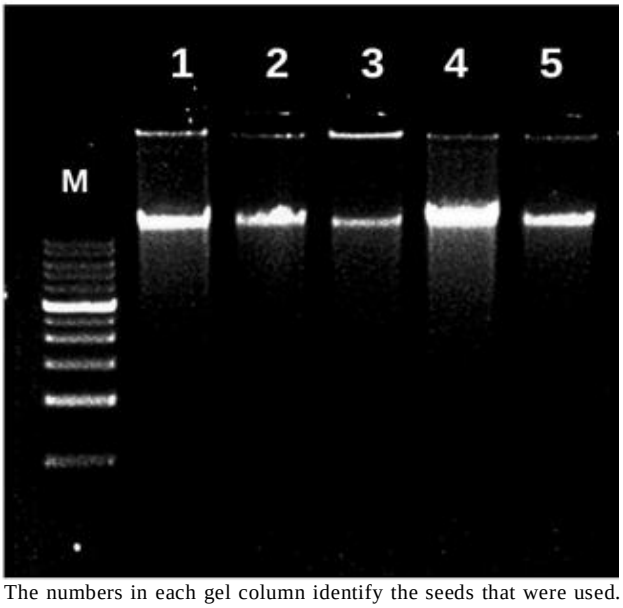
A) 5-7: Doyle and Doyle (1990); 8-10: Wizard Genomic DNA Purification Kit (Promega); B) 24-27: EasyPure Plant Genomic DNA Kit (TransGen); C) 12-15: Wizard PVP - Costa *et al*. (2015); 17-19: Ahmad, Ferguson and Southwick (2003); 22-23: CTAB-PVP – Costa *et al*. (2015); D) 28-30: Quick-DNA Plant/Seed Miniprep Kit (Zymo). M: 1Kb molecular weight marker (Promega)

Validating the DNA extraction protocol in seeds of the Cerrado cashew

The Wizard Genomic DNA Purification Kit (Promega) was the protocol used to extract DNA from the BGC 676 accession, as it presented the best cost/benefit ratio. The results for total DNA and quantification of the five samples extracted from the BGC 676 accession are shown in Figure 4 and Table 4, respectively, where it was possible to visualise the DNA bands for each of the samples. The quality of the DNA, as indicated by the 260/280 and 230/260 ratios, was also not a limiting factor for subsequent use in techniques that use molecular markers.

The viability test of the DNA extracted from each seed was carried out using PCR with an ISSR-type marker (I848). Amplification occurred in all of the samples (Figure 5), showing that the material can be used for subsequent analyses, such as genetic divergence.

Figure 4 - Result of DNA extraction from the BGC 676 accession of *Anacardium occidentale* L. using the Wizard Genomic DNA Purification Kit (Promega)

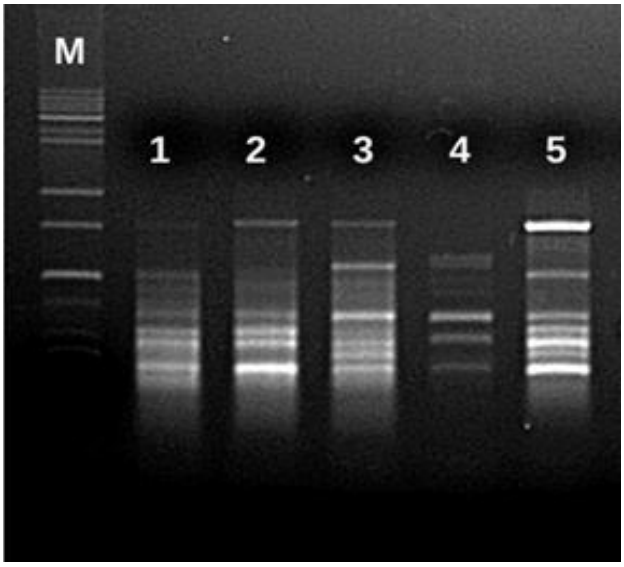


The numbers in each gel column identify the seeds that were used. M: 1Kb molecular weight marker (Promega)

Table 4 - Quantification of the DNA from the BGC 676 accession of *Anacardium occidentale* L. using the NanoDrop 2000 (Thermo Scientific)

ID	Concentration (ng/µL)	260/280	260/230
01	157.7	1.81	1.6
02	137.6	1.77	1.56
03	118.9	1.58	0.8
04	111.5	1.68	1.24
05	65.2	1.71	1.24

Figure 5 - Result of PCR with the ISSR 848 primer for DNA extracted from seeds of the BGC 676 accession of *Anacardium occidentale* L. to validate the extraction methodology



The numbers in each gel column identify the seeds that were used. M: 1 Kb molecular weight marker (Promega)

CONCLUSION

The methodology developed for DNA extraction from the endosperm of cashew seeds is non-destructive and effective. Cementitious adhesive is the recommended type of sealant, allowing a greater number of perforated seeds to germinate. The commercial Wizard® Genomic DNA Purification Kit (Promega) and EasyPure® Plant Genomic DNA Kit (TransGen) are the most suitable for extracting DNA from samples of cashew endosperm for use in PCR techniques based on molecular markers.

ACKNOWLEDGEMENTS

This study was funded by a research grant from the Empresa Brasileira de Pesquisa Agropecuária (Embrapa). The authors would like to thank Cândida Hermínia Campos de Magalhães, Ana Cecília Ribeiro de Castro and Ingrid Bernardo de Lima Coutinho for reviewing the initial version of the manuscript. The authors would also like to thank Adriano Lincoln Albuquerque Mattos for making the steel tubes used to collect the samples.

REFERENCES

AHMAD, R.; FERGUSON, L.; SOUTHWICK, S. M. Identification of pistachio (*Pistacia vera* L.) nuts with microsatellite markers. **Journal of the American Society for Horticultural Science**, v. 128, n. 6, p. 898-903, 2003.

BRAINER, M. S. C. P.; VIDAL, M. F. **Cajucultura nordestina em recuperação**. Fortaleza: Banco do Nordeste do Brasil, ano 3, n. 54, nov. 2021.

BURLE, M. L. **Conservação de recursos genéticos vegetais na Embrapa–histórico e perspectivas futuras**. Brasília: Embrapa Recursos Genéticos e Biotecnologia, 2019. 14 p.

CASTRO, A. C. R. Banco Ativo de Germoplasma de Cajueiro: Embrapa Agroindústria Tropical. *In*: PÁDUA, J. G.; ALBUQUERQUE, M. S. M.; MELO, S. C. M. (ed.). **Bancos e coleções de germoplasma da Embrapa**: conservação e uso. Brasília, DF: Embrapa Recursos Genéticos e Biotecnologia, 2020. 167 p.

CORREIA, S. J.; DAVID, J. P.; DAVID, J. M. Metabólitos secundários de espécies de Anacardiaceae. **Química Nova**, v. 29, p. 1287-1300, 2006.

COSTA, J. *et al*. Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols. **Food Chemistry**, v. 187, p. 469-476, 2015.

CRUZ, C. D. Genes Software – extended and integrated with the R, Matlab and Selegen. **Acta Scientiarum. Agronomy**, v. 38, n. 4, p. 547-552, 2016.

DOYLE, J. J.; DOYLE, J. L. Isolation of plant DNA from fresh tissue. **Focus**, v. 12, p. 13-15, 1990.

DWININGSIH, Y.; RAHMANINGSIH, M.; ALKAHTANI, J. Development of single nucleotide polymorphism (SNP) markers in tropical crops. **Advance Sustainable Science, Engineering and Technology**, v. 2, n. 2, p. 1-13, 2020.

FALEIRO, F. G. *et al*. O gênero *Passiflora*: diversidade, conservação e uso. *In*: FALEIRO, F. G. *et al*. (ed.). **Banco de germoplasma de *Passiflora* L. 'Flor da Paixão'**: caracterização fenotípica, diversidade genética, fotodocumentação e herborização. Brasília, DF: Pro Impress, 2020. 140 p.

IBGE. **Produção de castanha-de-caju (cultivo)**. 2023. Disponível em: https://www.ibge.gov.br/explica/producao-agropecuaria/castanha-de-caju-cultivo/br. Acesso em: 18 abr. 2024.

MA, Z. *et al*. A new, harmless, high-throughput endosperm-based DNA extraction method for wheat. **Ciência Rural**, v. 49, n. 9, p. 1-10, 2019.

NOVAES, T. E. R.; NOVAES, A. S. R. Análise dos potenciais medicinais do cajueiro (*Anacardium occidentale* Linn.): uma breve revisão. **Research, Society and Development**, v. 10, n. 1, p. 1-7, 2021.

OLIVEIRA, J. S. *et al*. Recursos genéticos e caracterização morfo-agronômica. *In*: FALEIRO, F. G. *et al*. (ed.). **Banco de germoplasma de *Passiflora* L. 'Flor da Paixão'**: caracterização fenotípica, diversidade genética, fotodocumentação e herborização. Brasília, DF: Pro Impress, 2020. 140 p.

PAIVA, S. R. *et al*. Caracterização de recursos genéticos. *In*: PAIVA, S. R. *et al*. (ed.). **Recursos genéticos**: o produtor pergunta, a Embrapa responde. Brasília, DF: Embrapa Recursos Genéticos e Biotecnologia, 2019. 298 p.

SILVA, M. N. Extração de DNA genômico de tecidos foliares maduros de espécies nativas do cerrado. **Revista Árvore**, v. 34, n. 6, p. 973-978, 2010.

VON POST, R. *et al*. A high-throughput DNA extraction method for barley seed. **Euphytica**, v. 130, p. 255-260, 2003.

ZHENG, X. *et al*. Non-destructive high-throughput DNA extraction and genotyping methods for cotton seeds and seedlings. **BioTechniques**, v. 58, n. 5, p. 234-243, 2015.