Journal of Tropical Biodiversity and Biotechnology
Volume 10, Issue 02 (2025): jtbb12766
DOI: 10.22146/jtbb.12766

Research Article

A Streamlined Plant DNA Extraction Method with Liquid Nitrogen-Free Approach

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Keywords:
Free liquid-nitrogen
DNA suitability
DNA quality
PCR
*rbcL*a
Submitted:
03 April 2024
Accepted:
11 December 2024
Published:
18 April 2025
Editors:
Furzani Binti Pa'ee
Sri Nopitasari

ABSTRACT

Molecular technique such as Polymerase Chain Reaction (PCR) is essential in various research fields. The amplification process of plant DNA can be challenging due to the presents of metabolites that can inhibit the polymerase eznyme, as well as expensive procedures or time-consuming laboratory work required. Most of the protocol involved liquid nitrogen, which is not always accessible, especially in laboratories with limited resources. Consequently, this study proposed an alternative protocol free from liquid-nitrogen usage, that was designed to be efficient in the DNA extraction from dry and fresh leaf samples across 40 plant species belonging to 27 different families. The DNA obtained from all the samples showed concentrations greater than 50 ng μL^{-1}, with the quality indexes in the acceptable range (A260/280: 1.50-2.21, A260/230:0.60-2.20). The efficacy of this method was demonstrated by successful PCR amplification using *rbcL* primer, validating the DNA suitability. This protocol can be considered a good option to be used both with fresh and dried plant leaves. Moreover, the absence of liquid nitrogen usage in the protocol could decrease the laboratory cost considerably and turning it into a more easily replicable method to be used in laboratories with limited resources.

How to cite:
Espinosa de los Monteros-Silva, N.E. et al., 2025. A Streamlined Plant DNA Extraction Method with Liquid Nitrogen-Free Approach. *Journal of Tropical Biodiversity and Biotechnology*, 10(x), jtbb12766. doi: 10.22146/jtbb.12766

INTRODUCTION

Molecular techniques play crucial role in various research fields, including genetic diversity, genetic mapping, evolutionary, breeding, and population studies (Demeke & Jenkins 2010; Tamari & Hinkley 2016). The initial step involves obtaining genetic material such as DNA or RNA in optimal quantity and quality for amplification process by Polymerase Chain Reaction (PCR) is very essential. However, obtaining high-quality genetic material from plants can be challenging due to the presence of natural metabolites that can inhibit the PCR techniques (Abdel-Latif & Osman 2017). Consequently, the effectiveness of the DNA extraction process has often been a limiting factor (Abdel-Latif & Osman 2017). Normally, DNA extraction follows three main steps i.e.: (1) membrane disruption; (2) separation and purification of DNA from other components such as proteins and lipids; and (3) concentration and purification of DNA (Shetty 2020). Yet, the specific reagents and techniques employed in each step may vary depending on the type of sample being analysed. In the case of samples derrived from plants, there are different factors that must be considered in DNA extraction protocol standardisation such as the presence of secondary metabolites , including high amounts of polyphenols and polysaccharides (Csaikl et al. 1998; Demeke & Jenkins 2010; Shepherd & McLay 2011; Chabi Sika et al. 2015; Inglis et al. 2018; Aboul-Maaty & Oraby 2019). They can interfere the DNA activity and the subsequent PCR analyses (Shepherd & McLay 2011). Because of these potential inhibitors, several protocols are developed using CTAB buffer along high salt concentrations, preventing the solubility of polysaccharides (Rezadoost et al. 2016). Meanwhile, polyvinylpyrrolidone (PVP) is used to be added to remove polyphenols by breaking the bond between DNA, RNA and phenolics, preventing loss of DNA and increasing DNA yield (Labra et al. 2001; Rezadoost et al. 2016; Abdel-Latif & Osman 2017). As well, the addition of β-mercaptoethanol has been used helping to reduce tannins and other polyphenols (Hwang & Kim 2000; Labra et al. 2001; Marín et al. 2021). In addition, many protocols indicate the use of phenol to separate cellular molecules and debris from the DNA, however, it requires special containment tools to avoid risks for the user and the environment (Sahu et al. 2012). Therefore , DNA extraction protocols are very often adjusted to particular plant species or plant tissues to obtain high-quality genetic material for downstream analyses (Csaikl et al. 1998; Shepherd & McLay 2011; Chabi Sika et al. 2015; Inglis et al. 2018; Aboul-Maaty & Oraby 2019; Marín et al. 2021). In this work, because of the more common availability, only β-mercaptoethanol was chosen as a good enough tool to avoid contaminants in the DNA isolation procedure.

There are specific commercial kits for plant DNA extraction however, they usually represent high costs, and even the traditional procedures include reagents that are not very common in laboratories with limited resources, adding the fact that they occasionally show limitations in terms of the amount of DNA obtained. Moreover, for plant tissue samples, most of the previous methods involve the use of liquid nitrogen at the first step of the DNA extraction process, not only to break cell but also as a good strategy to deactivate harmful enzymes preventing DNA damage (Porebski et al. 1997; Sharma et al. 2002; Kotchoni et al. 2011; Healey et al. 2014; Mayjonade et al. 2016; Afshar-Mohammadian et al. 2018; Lear et al. 2018; Serna-Domínguez et al. 2018; Quiñones et al. 2024). This reagent is well known because of its efficient processing of specimens, such as animals, fungi, and microorganisms samples (Rogers & Bendich 1988; Höss & Pääbo 1993; Lee et al. 2003; Jan Kieleczawa 2006; Varma et al. 2007; Asghar et al. 2015; Peñafiel et al. 2019). However, it is hazardous and expensive (Scharf et al. 2020) and its storage requires specific conditions that unavailable in most laboratories. Despite give the advantages, the use of liquid nitrogen may not be universally applicable or

replicable, especially in laboratories with limited resources.

Nowadays, there are numerous DNA protocols developed in the interest of obtaining suitable DNA by reducing costs and time. Determining "the best protocol" is strenuous and unfair, as each method has its own advantages and limitations depending on the research conditions (Mace et al. 2003; Hollingsworth et al. 2009; Demeke & Jenkins 2010; Zhang et al. 2010; Yanisko et al. 2011; Ahari et al. 2012; Fazekas et al. 2012; Hasan et al. 2012; Sahu et al. 2012; Jinlu et al. 2013; Parveen et al. 2016; Tamari & Hinkley 2016; Abdel-Latif & Osman 2017; Esfandani-Bozchaloyi et al. 2019; Paul et al. 2019). Even though there is a wide variety of standardised protocols available, most of them are limited to specific experimental units tailored for their projects. These protocols constraints a challenge when it used for different species, especially for the next-generation sequencing analysis that required high quality and quantity DNA from many different taxa simultaneously (Särkinen et al. 2012; Shetty 2020). In addition, the PCR analysis should be performed for assesing the DNA suitability. The amplification process using barcoding primers is one of the methods that could be used to validate the purity of DNA, especially since the basis for this study is species molecular identification, particularly when it is focused on unstudied areas, by supplementing the morphological techniques (Kool et al. 2012; Bezeng et al. 2017; Pathak et al. 2018; Amin et al. 2020; Mavrodiev et al. 2021). In plant study, approximately 2000 species have been described by molecular (barcoding) and morphological techniques every year (Prance 2001; Ng'ang'a 2019) and many of them were not clearly described in terms of distribution, ecology, threats, and benefits (Bebber et al. 2010; Ruas et al. 2022). For barcoding purposes, the *rbcL* plastid region has already reported as an important region to be used, which after the evaluation of several markers was adopted by the Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL) as one of the standard plant DNA barcodes, not excluding the use of supplementary markers that could be required depending on the aim of the research (Quandt et al. 2004; Anderson et al. 2006; Kress & Erickson 2007; Pirie et al. 2007; Taberlet et al. 2007; Haider 2011; Hollingsworth et al. 2011; Vere et al. 2015; Harnelly et al. 2018; Volenzo & Odiyo 2020; Ho et al. 2021).

Consequently, it is key to look for a feasible and inexpensive plant DNA extraction method that can be used as widely as possible. Considering common protocols, their advantages, and disadvantages, this research achieved its aim of showing a successful standardised protocol without liquid nitrogen, RNAse and PVP, for plant DNA extraction, suitable for PCR amplification in molecular identification activities, reducing the use of expensive or not commonly reagents available in limited resources laboratories.

MATERIALS AND METHODS

Genetic materials

The plant samples of 40 different species were collected in Napo Province, Eastern Ecuador. For lab analyses, as many as 2 to 4 healthy-young leaves per species were used in this study, being sure that at least 1 cm^2 per two leaves were available. Half of the total collected leaves for each species were stored in labelled Ziploc bags and frozen at -20 ºC using ice packs. the remaining leaves were stored in paper envelopes with silica gel for at least seven days, to be used as dry material. The samples were transported to the Molecular Biology and Biochemistry Laboratory of Universidad Regional Amazónica Ikiam for further analysis. Samples were collected under MAATE -ARSFC-2022-2415 and MAATE-CMARG-2023-0833 permits.

DNA Extraction

To isolate the total DNA content from leaves (fresh and dry leaves) the protocol consisted of several steps, avoiding the use of liquid nitrogen, polyvinylpyrrolidone (PVP) and RNase, i.e.

(1) Grind ~1 cm2 of leaves sample with 800-1000 µL CTAB 2 % (w v-1) (Solvent: 0.1M Tris HCl, 1.4M NaCl, and 0.02M EDTA pH 8) using a sterile mortar and pestle. Transfer the mix to a labelled 1.5 mL microtube.
(2) Add 8-10 µL of β-mercaptoethanol and barely mix by vortex for 5 seconds. Then, a spin to get down any drop from tube walls.
(3) Incubate the samples for 60 minutes at 60 °C. Stir gently every 15 minutes.
(4) Lay down the sample at room temperature for 2 minutes.
(5) Add 500 µL of chloroform: isoamyl alcohol (24:1) solution. Mix gently for 10 seconds by a vortex.
(6) Centrifuge during 15-20 min at 15 000 rpm.
(7) Transfer the supernatant (~600µl) to a newly labelled 1.5 mL microtube and add chilled isopropyl alcohol (~600 µl) considering a comparison of 1:1 (supernatant: isopropyl alcohol). Mix the samples by inversion.
(8) Incubate the samples for 30 minutes at -20 ºC.
(9) Centrifuge for 10 minutes at 10 000 rpm and carefully discard the supernatant (liquid phase) by inversion.
(10) Add 600 µL of 70 % ethanol and stir by inversion until seeing the pellet is floating.
(11) Centrifuge for 10 minutes at 5000 rpm and carefully completely discard the ethanol.
(12) Repeat steps 10 and 11 until you get clear ethanol in the tube.
(13) Let samples dry overnight at room temperature to eliminate every alcohol residue. NOTE: Dry process can be improved by letting the inverted tube on a sterile paper towel overnight.
(14) Finally, elute the DNA using 50-100 µL of ultrapure water and stir gently.

The quantity and quality of the DNA extracted were evaluated using spectrophotometer Nanodrop ND-ONEC-W (Thermo Scientific™) using 2 µL of each sample. The total DNA concentration was measured at 260 nm, and its quality was registered considering 50 µL of dilution factor and the comparison between A260/230 and A260/280. Also, an agarose gel electrophoresis of the total DNA was performed to estimate the size of molecules and the integrity of the genetic material obtained.

DNA suitability assay

In order to verify the suitability of the DNA extracted in activities such as molecular identification, the amplification of the *rbcL*a region was performed by PCR technique. A reaction of 10 µL as final volume was prepared using 1 µL of 10X Buffer, 0.8 µL of 25 mM $MgCl_2$, 0.2 µL of Taq, 2 µL of 10 mM dNTPs, 0.8 µL of each 10 µM primers (*rbcLaF:* 5´-ATGTCACCACAAACAGAGACTAAAGC 3´; *rbcLaR:* 5´-GTAAAATCAAGTCCACCRCG 3´), and 1 µL of 50ng μL^{-1} DNA template. PCR was performed with an initial temperature of 94 ºC for 2 minutes, followed by 35 cycles, consisting of an initial denaturation of 93 ºC for 1 minute, an annealing temperature of 55 ºC for 45 seconds, and an extension of 72 ºC for 1 minute. At the end of cycles, a final extension temperature of 72 ºC for 10 minutes was applied. The PCR products were then separated using 2 % agarose electrophoresis.

Data analysis

Analyses were conducted using R software (Version 4.3.3). First, density plots and Levene's tests were employed to assess the distribution and homogeneity of the datasets (DNA concentration and quality indexes), respectively. Mann-Whitney U test (for non-normally distributed datasets) was performed to compare the DNA concentrations and quality indexes between the two types of sample material (fresh and dry leaves).

Subsequently, multivariable Generalized Linear Models (GLMs) were used to examine the effects of sample-material (fresh and dry leaves) and plant family on the DNA concentration and quality indexes. Data transformation techniques such as logarithmic, square root, and inverse, were employed to achieve the best fit before the analysis. GLM with Gamma distribution using a logarithm link function was performed and, Q-Q plots of model residuals were used to test the conformity between the empirical vs. the given theoretical distribution.

RESULTS AND DISCUSSION

The implementation of an efficient DNA extraction process is imperative to achieve optimal results in terms of quantity and quality. The DNA extraction process is essential to achieve good amplification that lets the researchers study its genetic content. The DNA concentration needed for a successful PCR assay depends on the minimum requirements of the polymerase used (Bartlett & Stirling 2003). It is commonly in a range of 5-50 ng μL^{-1} for genomic DNA, and 0.1-1 ng μL^{-1} for plasmids, considering a final volume of PCR reaction of 50 µL (Steitz 1998; Bartlett & Stirling 2003; Van Pelt-Verkuil et al. 2008). The protocol performed in this study showed good DNA concentrations obtained from fresh and dry leaves of 40 plant-samples of 27 different families, collected in Napo Ecuador. The DNA concentration was greater than 50 ng μL^{-1} for all samples (Table 1).

Moreover, the data analysis showed no statistically significant differences in DNA concentration (p=0.93) between the two sample types (fresh and dry) (Table 2). In addition, GLMs analysis determined a non-significant impact of the type of material (***Pr (>Chisq)*=0.99**), but a significant impact of the plant family factor (***Pr (>Chisq)*=0.004**) in the DNA concentration (Table 3).

Furthermore, the quality indices (A260/A280 and A260/A230) of fresh and dry leaves showed statistically significant differences between them (Table 2), which was supported by GLMs analysis that demonstrated the significant influence of type material on A260/A280 and A260/A230 values (Table 3).

On the other hand, the plant family demonstrated a significant impact on the A260/A230 index (***Pr (>Chisq)***=0.03), however, it did not exhibit a significant effect on the A260/A280 quality index (***Pr (>Chisq)***=0.66) (Table 3). Additionally, the Q-Q plots employed depicted that the residuals of our models are aligned well with the expected theoretical distribution, as seen in Figure S1.

It is important to mention that the fact that DNA quantity was above 50 ng μL^{-1} for all samples (Table 1), instead of a problem just means that a previous DNA dilution step is necessary to reach a good PCR, and more DNA concentration is available if other assays are planned. Also, the total yield of DNA was higher than the reported in another study that consider DNA extraction processes based on CTAB (Mavrodiev et al. 2021).

Regarding the DNA quality, the ratio of A260/280 is commonly used considering an ideal range value of 1.8-2.0 (Hollingsworth et al. 2009; Desjardins & Conklin 2010; Pereira et al. 2011; Abdel-Latif & Osman 2017). This parameter also showed no statistically significant differences between

Table 1. Quantity and quality of DNA measured by spectrophotometry (Nanodrop ONE) in dry and fresh leaves of 40 plant-samples of 27 different families, collected in Napo Ecuador.

No.	Samples					Fresh leaves						Dry leaves					
	Plant family	Scientific name	Order	Spanish common name	English common name	Concentration (ng µL⁻¹)*	±SD	A260/280*	±SD	A260/230*	±SD	Concentration (ng µL⁻¹)*	±SD	A260/280*	±SD	A260/230*	±SD
1	Acanthaceae	*Fittonia albivenis* (Veitch) Brummitt.	Lamiales	Tortuga roja	Red Nerve Plant	323,04	215,36	2,04	0,12	1,35	0,13	752,89	286,32	1,91	0,04	1,51	1,03
2	Amaranthaceae	*Iresine herbstii* Hook.[a]	Caryophillalles	Molleja	Bloodleaf	489,89	157,99	2,05	0,07	1,55	0,48	1250,64	1377,84	1,69	0,49	2,03	0,08
3	Anacardiaceae	*Mangifera indica* L.[a,b]	Sapindales	Mango	Mango	576,60	412,29	2,11	0,08	1,96	0,30	285,04	92,53	2,10	0,01	1,83	0,04
4	Aquifoliaceae	*Ilex guayusa* Loes.[a]	Aquifoliales	Guayusa	Guayusa	520,78	217,91	2,07	0,01	1,97	0,11	573,84	231,63	1,79	0,49	1,62	1,00
5	Araceae	*Monstera deliciosa* Liebm.[a,b]	Alismatales	Costilla de Adán	Swiss-cheese Plant	1095,60	1110,54	2,00	0,13	1,89	0,77	268,42	138,10	1,82	0,03	1,15	0,07
6	Asparagaceae	*Cordyline fructicosa* (G.Forst.) Endl.[a]	Asparagales	Banderilla	Ti plant	400,10	34,56	1,86	0,25	1,85	0,52	802,13	683,56	1,81	0,34	0,60	0,07
7	Bignoniaceae	*Crescentia cujete* L.[a,b]	Lamiales	Pilche	Calabash tree	927,42	896,88	1,76	0,48	1,63	0,58	462,13	215,04	1,88	0,30	1,32	0,48
8	Bixaceae	*Bixa orellana* L.[a,b]	Malvales	Achiote	Lipstick-tree	171,92	142,84	2,02	0,04	2,00	0,27	522,72	321,60	1,82	0,22	1,70	0,26
9	Caricaceae	*Carica papaya* L.[a]	Brassicales	Papaya	Papaya	711,62	13,89	2,16	0,05	2,10	0,23	1127,96	697,42	2,10	0,06	1,92	0,09
10	Cyatheaceae	*Cyathea arborea* (L.) Sm.[a]	Cyatheales	Helecho gigante	West just treefern	1222,23	1795,67	2,15	0,08	1,90	0,32	216,02	90,03	1,75	0,23	1,48	0,85
11	Cyclanthaceae	*Carludovica palmata* Ruiz & Pav.[a]	Pandanales	Paja toquilla	Panama hat plant	542,48	183,10	2,05	0,04	1,96	0,34	336,17	138,75	1,87	0,07	1,05	0,19
12	Euphorbiaceae	*Codiaeum variegatum* (L.) A.Juss.[a]	Malphighiales	Croton	Croton	816,44	792,47	2,10	0,04	2,00	0,30	961,36	65,92	2,00	0,22	1,99	0,01
13	Euphorbiaceae	*Manihot esculenta* Crantz.[a,b]	Malphighiales	Yuca	Cassava	403,91	394,18	2,08	0,10	2,00	0,22	754,60	55,61	2,10	0,04	1,77	0,01
14	Euphorbiaceae	*Croton lechleri* Muell. Arg.[a]	Malphighiales	Sangre de drago	Dragon´s Blood	1787,72	1358,89	1,94	0,14	2,15	0,01	4438,61	379,84	1,97	0,01	1,61	0,01
18	Fabaceae	*Cedrelinga cateniformis* (Ducke) Ducke	Fabales	Chuncho	Tornillo	136,79	56,53	1,82	0,22	1,22	0,02	170,06	0,81	1,89	0,01	1,50	0,01
15	Fabaceae	*Mimosa pudica* L.[a]	Fabales	Dormilona	Sensitive plant	631,80	660,42	2,15	0,00	1,99	0,17	156,76	67,49	2,00	0,13	1,17	0,08

Table 1. Contd.

No.	Samples					Fresh leaves						Dry leaves					
	Plant family	Scientific name	Order	Spanish common name	English common name	Concentration (ng µL⁻¹)*	±SD	A260/280*	±SD	A260/230*	±SD	Concentration (ng µL⁻¹)*	±SD	A260/280*	±SD	A260/230*	±SD
16	Fabaceae	*Arachis pintoi* Krapov. & W.C.Greg.[b]	Fabales	Maní forrajero	Yellow peanut plant	255,34	120,97	2,03	0,04	1,76	0,61	202,64	47,57	1,50	0,38	1,58	0,29
17	Fabaceae	*Inga edulis* Mart.[a]	Fabales	Guaba	Ice-cream bean	170,57	180,69	1,99	0,20	2,10	0,04	286,05	156,32	2,10	0,01	1,78	0,54
19	Fabaceae	*Brownea grandiceps* Jacq.[a]	Fabales	Cruscaspi	Rose of Venezuela	263,61	173,57	2,16	0,03	2,00	0,29	486,59	178,00	2,10	0,10	1,79	0,02
20	Heliconiaceae	*Heliconia rostrata* Ruiz & Pav.[a,b]	Zingiberales	Heliconia lorito	Hanging Lobster's Claw	372,28	97,90	1,99	0,16	1,60	0,51	328,35	244,13	1,76	0,26	0,98	0,38
21	Lamiaceae	*Mentha × piperita* L.[a,b]	Lamiales	Menta panga (kichwa)	Peppermint	449,70	241,64	2,04	0,12	1,87	0,03	2105,34	1579,88	2,10	0,15	1,77	0,05
22	Lamiaceae	*Ocimum basilicum* L.[a,b]	Lamiales	Albahaca	Basil	123,53	98,20	2,02	0,28	1,95	0,01	327,65	281,19	1,84	0,05	1,03	0,37
23	Lauraceae	*Persea americana* Mill.[a,b]	Lamiales	Aguacate	Avocado	1132,32	1421,50	2,11	0,07	1,97	0,35	108,62	2,38	2,10	0,09	0,75	0,01
24	Malpighiaceae	*Banisteriopsis caapi* (Spruce ex Griseb.) C.V.Morton.[a]	Malphighiales	Ayahuasca	Soul vine (yage)	183,97	72,80	1,92	0,01	1,53	0,00	86,46	1,33	1,88	0,00	1,26	0,01
25	Malvaceae	*Theobroma cacao* L.[a]	Malvales	Cacao	Cacao	416,06	117,68	2,10	0,01	1,60	0,15	225,73	98,64	2,04	0,04	1,33	0,28
26	Malvaceae	*Theobroma bicolor* Humb. & Bonpl.[a]	Malvales	Cacao blanco	Macambo tree	141,48	5,55	2,02	0,07	1,77	0,36	183,81	42,21	2,03	0,01	1,86	0,44
27	Musaceae	*Musa x paradisiaca* L.[b]	Zingiberales	Plátano	Banana	449,53	135,23	2,20	0,01	1,53	0,67	253,48	204,98	1,71	0,50	1,07	0,28
28	Myrtaceae	*Eugenia stipitata*.[a]	Myrtales	Arazá	Arazá	304,56	95,59	2,11	0,01	2,02	0,26	200,40	66,86	2,10	0,02	1,69	0,47
29	Myrtaceae	*Syzygium malaccense* (L.) Merr. & L.M.Perry.[a,b]	Myrtales	Pumarosa	Malay apple	212,10	240,60	2,04	0,09	1,51	0,07	572,92	482,78	1,63	0,39	1,54	0,02
30	Myrtaceae	*Psidium guajava* L.[a,b]	Myrtales	Guayaba	Common guava	1413,80	1383,67	2,10	0,02	1,58	0,51	466,98	76,20	2,04	0,02	1,57	0,07

Table 1. Contd.

No.	Samples					Fresh leaves						Dry leaves					
	Plant family	Scientific name	Order	Spanish common name	English common name	Concentration (ng µL^{-1})*	±SD	A260/280*	±SD	A260/230*	±SD	Concentration (ng µL^{-1})*	±SD	A260/280*	±SD	A260/230*	±SD
31	Oxalidaceae	*Averrhoa carambola* L.[a,b]	Oxalidales	Carambola	Copoazu	611,98	314,47	2,14	0,03	2,20	0,29	1663,19	1213,98	2,13	0,04	2,08	0,04
32	Piperaceae	*Piper aduncum* L.[a]	Piperales	Matico	Spiked pepper	453,99	161,76	2,11	0,05	2,01	0,20	1311,74	758,55	2,12	0,02	1,80	0,69
33	Piperaceae	*Piper peltatum* L.	Piperales	Maria panga	Largeleaf fingertree	785,62	913,39	1,98	0,17	1,47	0,69	347,55	25,54	2,17	0,04	1,93	0,42
34	Rubiaceae	*Borojoa patinoi* Cuatrec.[a]	Gentianales	Borojo	Borojo	559,87	276,31	1,94	0,17	1,53	0,25	179,86	50,13	2,01	0,07	0,91	0,08
35	Rubiaceae	*Coffea arabiga*.[a,b]	Gentianales	Café	Coffee	133,90	51,96	1,68	0,23	1,13	0,06	163,90	51,96	1,82	0,23	1,40	0,03
36	Sapindaceae	*Nephelium lappaceum* L.[a,b]	Sapindales	Rambután/ Achotillo	Hairy Lychee	146,15	37,04	2,03	0,09	1,43	1,03	271,33	1,44	1,89	0,03	1,53	0,04
37	Sapotaceae	*Pouteria caimito* Radlk.[a,b]	Ericales	Avio	Star Apple	439,18	185,92	2,04	0,08	1,82	0,16	235,96	91,52	2,10	0,09	1,68	0,27
38	Solanaceae	*Solanum quitoense* Lam.[a]	Solanales	Naranjilla (cocona)	Lulo (Quechua)	226,42	141,14	2,02	0,15	1,93	0,65	829,40	289,08	2,05	0,04	1,69	0,14
39	Solanaceae	*Cestrum nocturnum* L.[a]	Solanales	Caballero de la noche	Night jessamine	540,84	302,46	1,98	0,21	1,84	0,61	970,96	242,55	1,95	0,13	1,87	0,70
40	Zingiberaceae	*Hedychium coronarium* J.Koenig.	Zingiberales	Lirio de arroyo	White ginger lily	239,16	64,74	1,97	0,10	1,13	0,16	263,09	112,26	1,99	0,07	1,79	0,34

*Mean of three replicates. [a] High content of polyphenols reported in literature.[b]High content of polysaccharides reported in literature.

Black text: Gymnosperms. Blue text: Angiosperms. Green highlighted: Eudicots. Yellow highlighted: Magnoliids. Salmon highlighted: Monocots. Blue highlighted: Pteridophyta

Table 2. Statistical differences in DNA extraction between fresh and dry leaves from 40 plant-samples of 27 different families, collected in Napo Ecuador

Variable	Mean Dry leaves ng μL^{-1} (± SD)	Mean Fresh leaves ng μL^{-1} (± SD)	*p-value*
Concentration	628 (± 765.73)	519.61 (± 380.57)	0.93
A260/A280	1.94 (± 0.16)	2.02 (± 0.11)	0.02*
A260/A30	1.52 (± 0.37)	1.77 (± 0.28)	0.002**

Significance: p < 0.1: '*', p < 0.01: '**', p < 0.001: '***'.

Table 3. Statistical significance of the impact of the type of material (fresh and dry leaves) and plant family on DNA quantity and quality of 40 plant-samples of 27 different families, collected in Napo Ecuador.

Variable	Data transformation	Factor	DF	Chisq	*Pr (>Chisq)*
Concentration	Log10	Material	1	1.22	0.99
		Plant Family	26	2.89	0.004 **
A260/A280	Inverse	Material	1	6.61	0.008 **
		Plant Family	26	0.86	0.66
A260/A30	Sqrt	Material	1	3.13	0.0002 ***
		Plant Family	26	1.71	0.03 *

DF: Degrees of Freedom, Chisq: Chi-square statistical value, Pr(>Chisq): P-value associated with Chi-squared statistical value. Significance: p < 0.1: '*', p < 0.01: '**', p < 0.001: '***'. All variables were treated by multivariable GLM with a Gamma distribution (Log link function).

the type of material (Table 2), and the values acquired for this index were from 1.50 to 2.20, which was expected because of the different possible metabolites present in samples depending on the plant families. For fresh material, twenty five samples were placing just in the ideal range, and fifteen showed values lightly above this range (until 2.20), which suggest the presence of RNA (Scientific 1975; O'Neill et al. 2011; Matlock 2015). It should happen because the protocol presented in this work avoided the use of RNAse, which despite of its importance and efficiency, can be uncommon in laboratories with limited resources.

On the other hand, for dry material, twelve samples showed ideal values, five samples can still be labelled as "clean DNA" (>1.7) (O'Neill et al. 2011). Moreover, only two samples were below 1.7 with the lowest value at 1.50 for *Arachis pintoi*, which can be related to its naturally high-protein nature molecule content (Hertentains & Ruiloba 2010; de Almeida Araújo et al. 2022) but also to the oxidation process of leaves when drying, which can result in the concentration of components identified as contaminants in the DNA quality evaluation by spectrophotometry (Sahu et al. 2012; Snoussi et al. 2021). It is also supported by GLMs analysis employed where the type of material demonstrated a relevant impact on the A260/280 ratio (***Pr (>Chisq)*** =0.008) (Table 3). In addition, one sample belonging to the gymnosperms group (*Cyathea arborea* (L.) Sm.) displayed a little lower A260/280 quality index in dry samples (1.48) than the values obtained by Jamaludin et al (2020) (1.90-1.99), who developed a specific CTAB-based protocol including polyvinylpyrrolidone (PVP) to reduce the problems of phenolics present in Cyatheales plant order (Sahu et al. 2012; Jamaludin et al. 2020). Yet, that is a clear example of a good protocol performed specifically for limited group of plant families, the opposite objective of this work which seeks to be global. Another important fact to take into account is that the accuracy of the spectrophotometer can slightly shift the values measured, especially for the A260/280 ratio where many times the 1 nm accuracy specification of equipment will result in ± 0.2 (Desjardins & Conklin 2010; Matlock 2015; Thermo Scientific 2020). It can also explain why some researchers do not take the ideal range as an entire strength and unbreakable parameter, instead sometimes work with values with few decimals of difference (Matlock 2015;

Thermo Scientific 2020). Although in spite of the absorbance at 280nm is a commonly used indicator of protein content, mainly due to tryptophan and tyrosin side chains coming from the sample (O'Neill et al. 2011), certain samples may be difficult to be successfully evaluated at that wavelength due to interference with ionic reagents used in the DNA extraction (O'Neill et al. 2011; Clark et al. 2015; Lucena-Aguilar et al. 2016). In those cases, it is recommended to evaluate the samples at a different wavelength such as 228nm as an alternative to identify the presence of peptide bonds (O'Neill et al. 2011).

Because of the reason exposed in the last paragraph, the other quality index (260/230) is often considered the more accurate index to estimate the DNA quality (O'Neill et al. 2011). Its value could indicate that organic compounds or chaotropic salts such as thiocyanate salt are in the DNA (Gupta et al. 2013; Zhong et al. 2013) and the ideal range is 2.0-2.2 (Thakuria et al. 2008; Antony-Babu et al. 2013; Santos et al. 2018). For this ratio, values obtained were lower than the 260/280 index, but all indices greater than 1.5 (Table 1) can be appraised as a guideline for good DNA quality (Desjardins & Conklin 2010). As well as in this protocol, variability and values below the ideal range for this index (2.0-2.2) are common in procedures with organic separation phases, since reagents such as phenol, guanidine HCl, carbohydrates and EDTA, commonly used in the DNA extraction process have absorbances close to ~230 nm (De Campos et al. 2017). Considering it, for this work, EDTA and carbohydrates can be related to a decrease of A260/230 values, the first one because it is part of the lysis buffer, however it was used for all samples and not all of them showed low values, so the second one reagent mentioned could be more related to it because it is often becoming problem in plant samples as a natural content (Scientific 1975; Matlock 2015).In contrast, values a little bit higher than the ideal range can be related to a slight variation in the pH, however, it is not always a problem because it does not necessarily act as a PCR inhibitor, and it is still considered an acceptable error range for this index (Wilfinger et al. 1997).

In general, the quality indexes obtained in the DNA extraction process are closely related to some factors such as the secondary metabolites present (Friar 2005; Sahu et al. 2012; Bailey et al. 2022). Its content is directly related to the plant family which by the data analysis employed in this study, revealed its great effect not only in the quality but also in the DNA concentration obtained (Table 3). Thus, our results can also be explained by the concentrations of β-mercaptoethanol (1 %), which is a reducing agent that breaks the disulfide bonds in the thiol groups of cysteine residues, altering the protein structure, eliminating ribonucleases released during cell lysis (Rezadoost et al. 2016; Spadoni et al. 2019; Schenk et al. 2023; Liu 2024). On the other hand, CTAB (2 %) is an amphipathic surfactant detergent that facilitates the lysis of both the cell wall and the plasma membrane (Križman et al. 2006; Krishnan et al. 2024). DNA extraction dissolves membrane molecules in an aqueous medium, forming micelles that encapsulate molecules such as phospholipids and proteins (Križman et al. 2006; Krishnan et al. 2024). Meanwhile, a high ionic strength caused using NaCl (1.4 M) promotes the formation of complexes between proteins and CTAB, allowing their removal during the separation of the organic phase (Heikrujam et al. 2020). Thereby, it facilitates the purification of DNA from polyphenols and proteins, so, a high concentration of those reagents can help to improve DNA extraction (Lodhi et al. 1994; Khanuja et al. 1999; Križman et al. 2006; Kool et al. 2012; Sahu et al. 2012; Arruda et al. 2017; Heikrujam et al. 2020; Krishnan et al. 2024). Additionally, it is interesting to mention that the DNA extraction of Laurales and Piperales (Magnolids), monocots, and eudicots have shown similar results in terms of purity (260/230: 1.8-2.0) with another procedure based on the CTAB method

by Doyle and Doyle (1987) utilizing a buffer with relatively large amounts of CTAB (3X) and sodium chloride (4M) (Mavrodiev et al. 2021). However, despite their methodology's decreased extraction time, it describes a protocol for use with EconoSpin All-In-One Silica Maxi Spin Columns (catalog no. 2040-050; Epoch Life Sciences, Missouri City, Texas, USA), which can be easily scaled for use with the Mini (Epoch 1910-050/250) or Midi (Epoch 050) Spin Columns by adjusting the amount of starting material and extraction buffer (Mavrodiev et al. 2021). In comparison, the protocol presented in this work showed similar results with fewer laboratory resources.

The elimination of any type of columns uses and equipment for automatic DNA extraction means that the difference in values obtained for DNA concentration and quality between samples could be closely associated with the skills of the human operator. So, it is important to be very careful in some steps such as the separation of the organic from the aqueous phase where we have to take supernatant (aqueous phase) that contains the DNA, leaving the interphase, lipids, and debris at the bottom of the tube (organic phase) (McKiernan & Danielson 2017; Heikrujam et al. 2020). If a little interphase or organic phase is recovered and mixed with the aqueous phase, the index value of A260/280 could decrease because of the organic compounds present, resulting in low-quality DNA. Also, to improve the value of the A260/230 ratio, despite the importance of ethanol in the washing steps of the protocol, it is crucial to be sure that all of it is removed from the DNA pellet because it also could be a strong PCR inhibitor (Schrader et al. 2012). In addition, we realise that using dry material yielded slightly higher mean values than fresh material for DNA concentration and quality indexes (Table 2).

On the other hand, the agarose gel electrophoresis of total DNA extracted showed thin and thick bands that act as an indicator of the molecular weight (Lee et al. 2012) (Figure 1 and Figure 2). For fresh material, we can identify a band above 10kb, which corresponds to genomic DNA, and marked pattern of two lower bands between 1k and 2k bp, which can correspond to RNA molecules such as 28S and 16S ribosomal, respectively (Figure 1). It is mentioned considering as a reference another study where the same pattern was identified after the use of the CTAB-LiCl extraction method (Vennapusa et al. 2020). The visualisation of these bands is possible because the method extracts the total nucleic acids from the tissue, as RNase is not used. Furthermore, a clear difference in DNA integrity between fresh and dry material (Figure 1 and Figure 2). Even fresh material displayed expected bands, many of them show smears, suggesting DNA degradation (Zhang et al. 2004; Schwessinger 2023). For dry material samples integrity observed was lower with a big smear in almost every sample (Figure 2). This fuzzy effect can be related to some factors including the presence of impurities in the sample such as high protein and salt concentrations, which can help to explain the results (Scientific 2022). However, it can also be closely related to DNA degradation because avoiding liquid nitrogen affects the good condition of the genetic material (Quiñones et al. 2024).

Nevertheless, all samples examined in this study demonstrated acceptable DNA concentration and quality indexes for both fresh and dry material (Table 2). It was obtained even with 34 of the 40 considered species having at least one report about their significant to high content of polyphenols, and, 15 of 40 which have reported their remarkable content of polysaccharides (Table 1) (Wollgast & Anklam 2000; Jiménez-Escrig et al. 2001; Torres et al. 2002; Sotelo et al. 2010; Thitilertdecha et al. 2010; Gómez et al. 2012; Sahu et al. 2012; Saffoon et al. 2014; Khanam et al. 2015; Gan et al. 2018; Hendawy et al. 2018; Olvera et al. 2018; Ahmed et al. 2019; Galviz-Quezada et al. 2019; Herrera-Calderon et al. 2019; Ijaz et al. 2019; Ribeiro de Sousa et al. 2019; Suleiman 2019; Gonçalves et al. 2020; Jamaludin et al. 2020; Lima et al. 2020; Savi et al. 2020; Spórna-Kucab et al. 2020; Asy'ari Hasbullah & Rini Umiyati 2021;

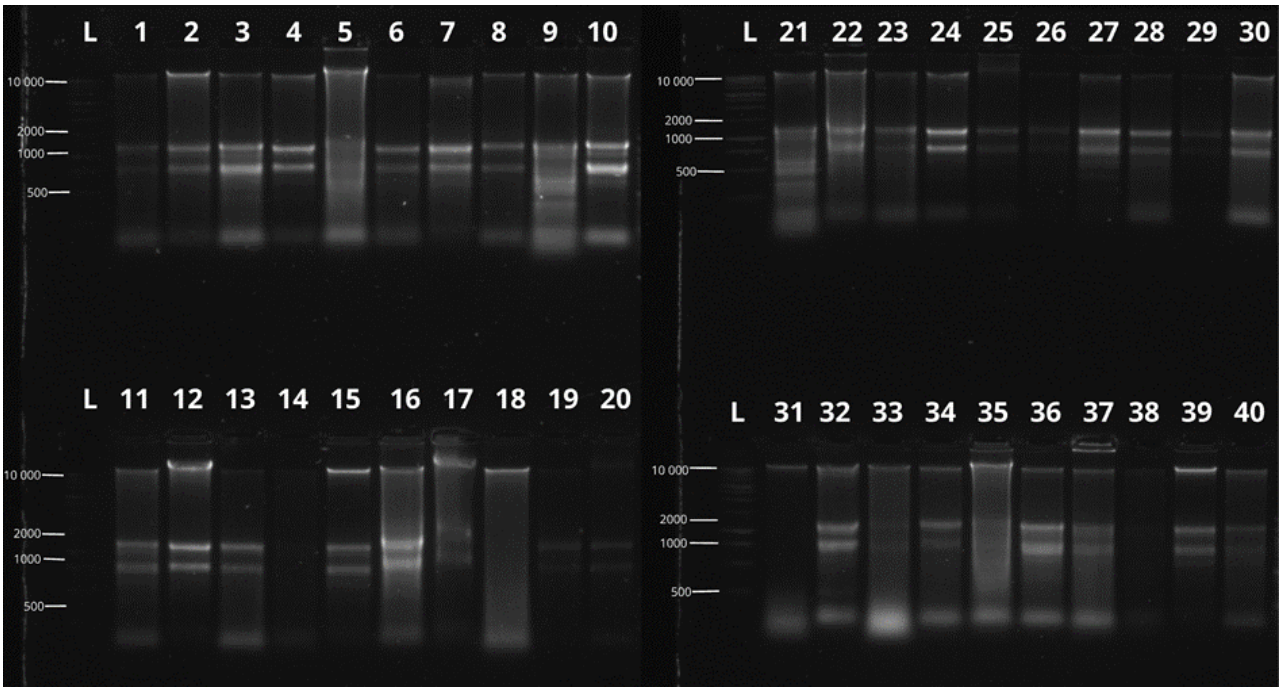

Figure 1. Agarose gel (1 %) electrophoresis of the genomic DNA extracted from fresh leaves. L: 1Kb ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

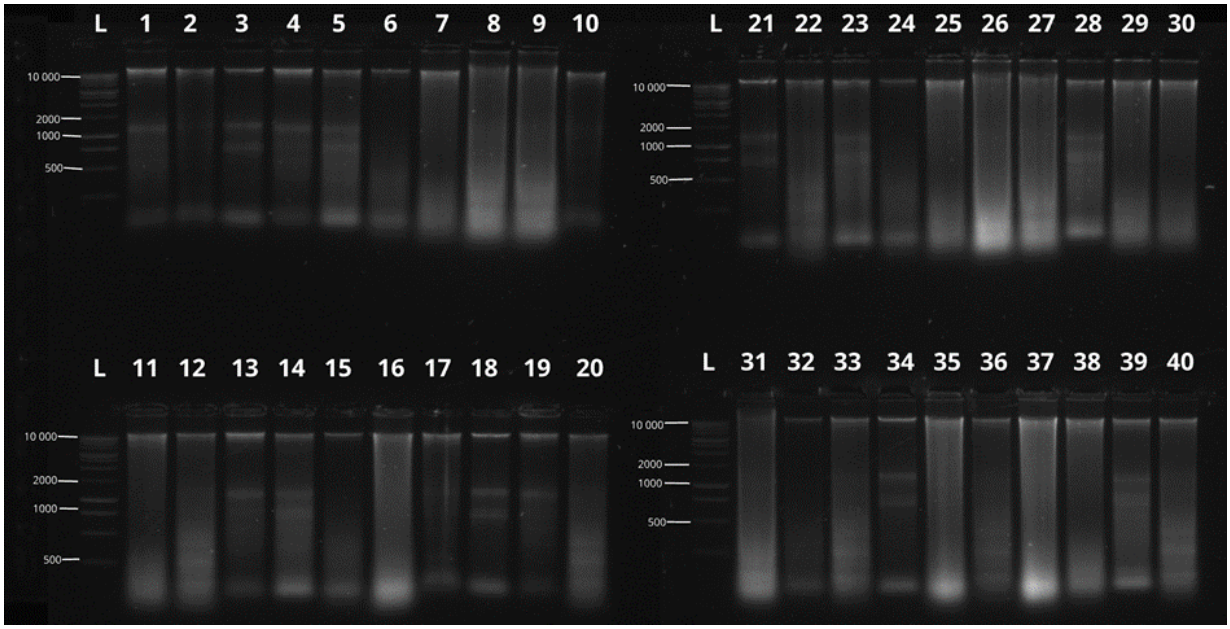

Figure 2. Agarose gel (1 %) electrophoresis of the genomic DNA extracted from dry leaves. L: 1Kb ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

Fernandes de Araújo et al. 2021; Nguyen Thi et al. 2021; Simpson et al. 2021; Abd Elkader et al. 2022; Haggag 2022; Korany et al. 2022; Dk et al. 2023; Kim & Iida 2023; Obregon et al. 2023; Song et al. 2023; Zhou et al. 2023). Furthermore, it is important to highlight the applicability of data analysis techniques like GLM, which in addition to its contribution to understanding how really variables affect, does not always necessitate the normality of predictors. It stands out in its utility in real-world biological data applications (Bolker et al. 2009; Keselman et al. 2016).

The success of the extraction method in obtaining good-quality genomic DNA can suggest its interesting applicability. It should be noted that PCR could be one of the keys to determining the suitability of the genetic material (Bolker et al. 2009; Schrader et al. 2012; Keselman et al. 2016; Handy et al. 2020). According to it, the PCR assay showed a successful amplification of

the *rbcL*a region for the total of samples without differences between dry and fresh material used, with a band of ~550pb as expected (Figure 3 and Figure 4).

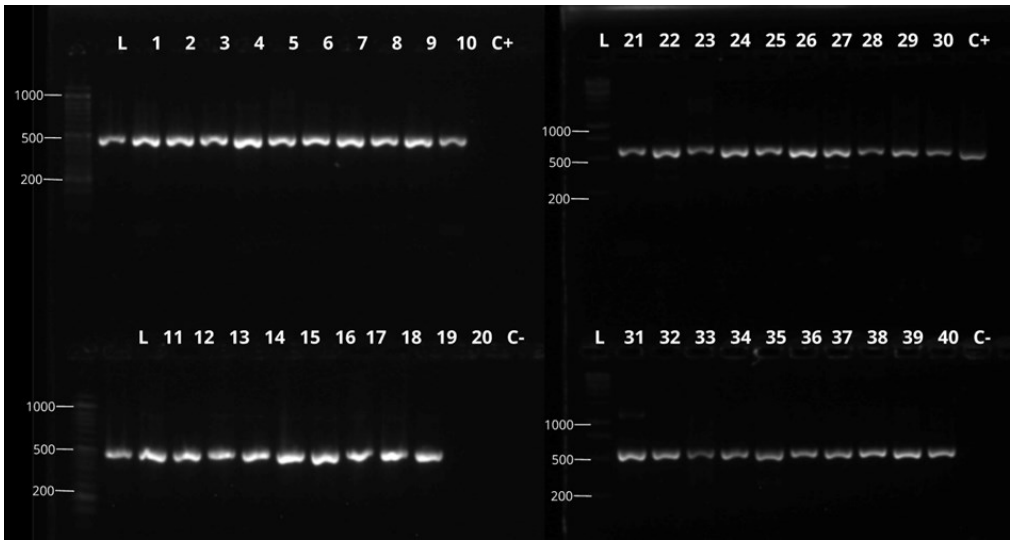

Figure 3. Agarose gel (2 %) electrophoresis of the amplification of *rbcL*a region using DNA from fresh leaves**.** L: 100bp ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

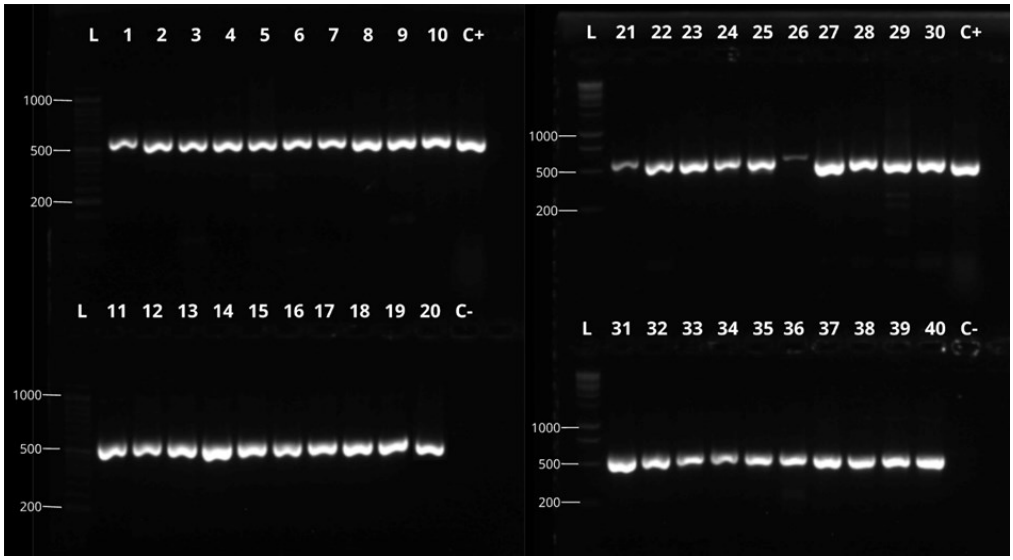

Figure 4. Agarose gel (2 %) electrophoresis of the amplification of *rbcL*a region using DNA from dry leaves**.** L: 100bp ladder. Wells are labelled with numbers (1-40) following the species listed in Table 1.

So, these results probe the suitability of DNA extracted for PCR activities in molecular plant species identification, suggesting that this protocol can be used for fresh or dry plant samples. Moreover, without using liquid nitrogen in the protocol, the cost of the DNA extraction process decreases considerably and turning it into a more easily replicable method to be used in laboratories with limited resources.

Finally, it is weighty to emphasise the importance of evaluate the efficiency of this procedure when using herbarium material or long storage samples, as well as evaluate its applicability to obtain DNA that can be uses for more complex techniques that involves enzymes restriction, Next Generation Sequencing (NGS) analysis, and the use of other type of molecular markers such as RFLPs or SSRs, in order to those methods can ask for more rigorous DNA requirements.

CONCLUSION

The protocol presented in this work demonstrated its applicability for molecular identification aims, and it can be useful in extracting DNA from at least 27 plant families, even groups of high phenolic content. We promote the application of this protocol for different purposes to evaluate its broad applicability. Meanwhile, the protocol is a good option, especially in academic fields and laboratories with limited resources.

AUTHOR CONTRIBUTION

NEDLMS and AOM designed the research. AOM and AAZ collected the plant material. NEDLMS, AOM, AAZ and JCC processed the samples until get results. NEDLMS and AOM analysed the data. NEDLMS, AOM, AAZ, JCC, EF, SL and MCP wrote the original draft and agreed to the final manuscript.

ACKNOWLEDGEMENTS

We express our gratitude to Andrea Sosa for her valuable assistance in providing feedback on this study. Lastly, we especially thank the BIOGEEC Project and SUNBOR GRANT from the Suntory Foundation for Life Sciences, which indirectly but significantly contributed to assessing the effectiveness of the DNA extraction protocol.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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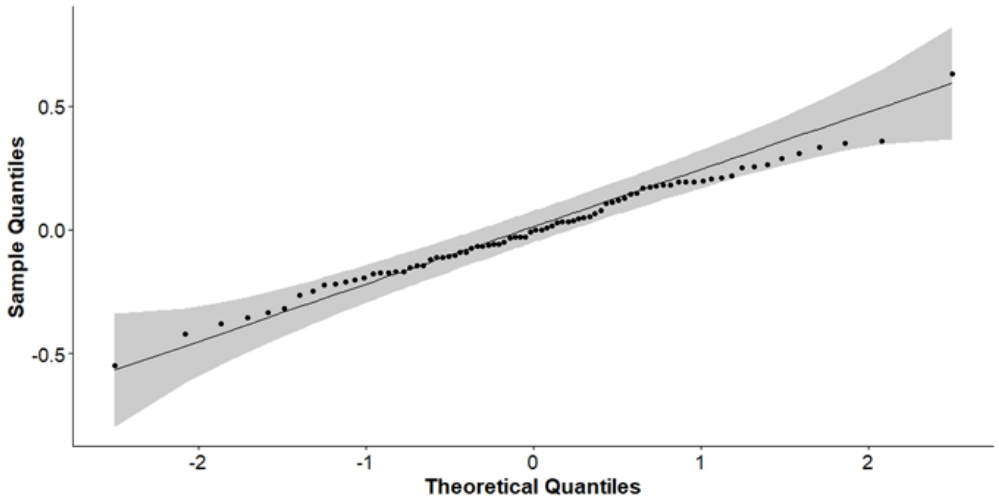
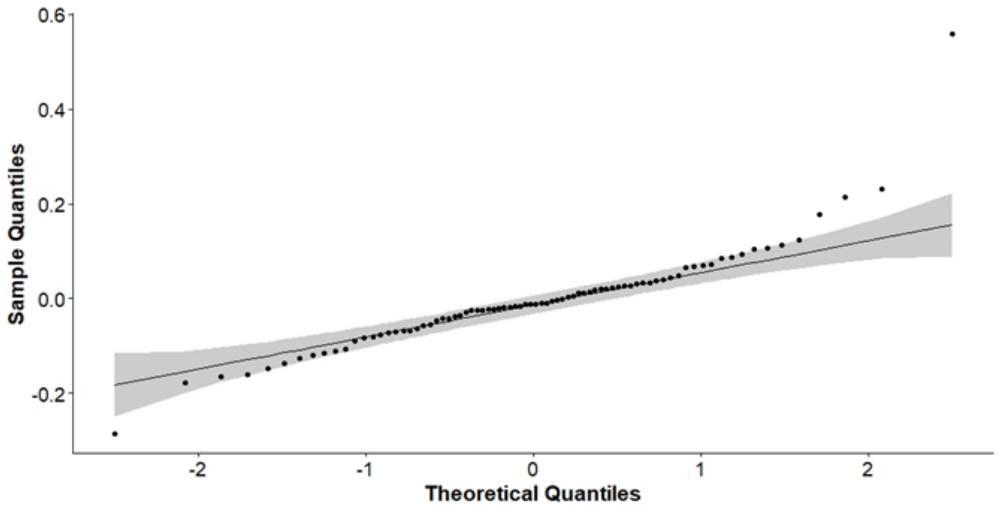
APPENDICES

A.



B.



C.

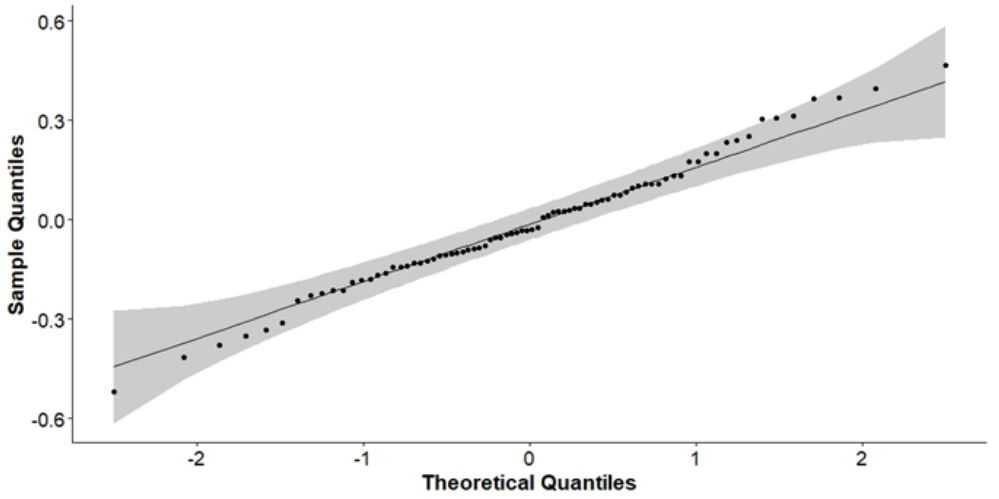


Figure S1. Q-Q Plots for residual models of DNA quantity and quality of 40 plant-samples of 27 different families, collected in Napo Ecuador. A: DNA Concentration Model. B: A260/280 Model. C: A260/230 Model.