Genomic DNA extraction and phenolic content of salty and tannic plant material of two mangrove tree species from the mexican pacific coast

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Abstract

Mangroves, unique tree and shrub species inhabiting coastal saline environments, exhibit distinctive ecological and morpho-physiological traits, including forming pure intertidal stands and possessing specialized mechanisms for salt excretion and aerial root respiration. These species produce valuable antioxidants, such as phenols and tannins, with significant pharmacological, ecological, and toxicological implications. This study aimed to develop a highly efficient DNA extraction protocol for mangrove leaves rich in salt and tannins. The commonly used CTAB extraction protocol and a commercial DNA extraction kit were modified to enhance DNA purity and yield. The antioxidant capacity of the samples was assessed using various protocols. The results demonstrated that the modified commercial kit outperformed other methods in extracting DNA from mangrove leaves, effectively overcoming challenges associated with high salt and phenolic compounds that could impede next-generation sequencing (NGS) analysis. Furthermore, the findings revealed an inverse relationship between tannin concentration and DNA extraction yield. This study offers a valuable resource for DNA extraction protocols from tannin-rich plant materials. In conclusion, the modified commercial kit represents the most efficient and effective approach for extracting DNA from tannin-rich mangrove leaves.

Keywords: *DNA yield, tannins, antioxidants, NGS analysis*

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Introduction

Mangroves are described as trees and shrubs that inhabit coastal environments with warm or tropical climates, where the coastline is flat, muddy, and with brackish waters, sometimes extending inland where the water loses its salinity (Charcape-Ravelo & Mourtarde, 2005). These plant species function as the ecotone that separates the coast from the mainland and exhibit unique ecological and morpho-physiological characteristics, such as their ability to form pure stands, their presence in intertidal environments, and their structure as trees or shrubs (Tomlinson, 1986; Saenger, 2002). Additionally, mangroves have unique adaptations to their environment, such as aerial roots that allow them to breathe and mechanisms to excrete excess salt from their bodies (Charcape-Ravelo & Mourtarde, 2005). The structure and productivity of mangroves are significantly affected by climatic conditions, resulting in biomass and primary growth variations (Cunill et al., 2022).

One notable trend is that productivity tends to decrease with increasing latitude (Woodroffe et al., 1988; Joshi & Ghose, 2014). Another environmental constraint is related to precipitation, which is widely acknowledged as a limiting factor in plant development and metabolism (Neale & Kremer, 2011). Specifically, mangrove species produce antioxidants such as phenols and tannins and other phytochemicals, including steroids, glycosides, triterpenes, saponins, flavonoids, and alkaloids. Substances like alkaloids, phenols, steroids, and terpenoids are secondary metabolites with toxicological, pharmacological, and ecological importance (Vinoth, 2019). On the other hand, antioxidants have the potential to treat diseases associated with reactive oxygen species, such as cancer, cardiovascular diseases, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, and aging (Valko, 2007). Plant species generally produce secondary metabolites with antioxidant capacity to protect themselves from other organisms or stressful conditions. Antioxidants absorb and neutralize free radicals, quench singlet and triplet oxygen, or break down peroxides, preventing oxidative damage to DNA, proteins, and other essential biomolecules.

These metabolites can facilitate or hinder processes and protocols for the extraction of genetic material, DNA, and RNA, as mentioned by Tahmasebi and Nasrollahi (2023), who compare different DNA extraction protocols for the genus *Halocnemum* with high concentrations of proteins, polyphenols, and other secondary metabolites. The CTAB (Cetyltrimethylammonium bromide) genomic DNA extraction protocol with modifications proposed by Cullings (1992), which aims to disrupt membranes and separate contaminants from nucleic acids, utilizes a chloroform-isoamyl alcohol mixture that separates contaminants in the organic phase and nucleic acids in the aqueous phase (Tamari et al., 2013). Moreover, this protocol has been widely used and adapted in various molecular studies of mangrove species worldwide, as demonstrated by Nettel and Dodd (2007), Nettel et al. (2008), Sahu et al. (2012), Nettel et al. (2013), Millán-Aguilar et al. (2016), and Tahmasebi and Nasrollahi (2023).

Furthermore, Aljanabi and Martínez (1997) and Wang et al. (2011) proposed alternative protocols for genomic DNA extraction. These protocols utilize molecular buffers and detergents to achieve high-quality DNA extraction with optimal purity and yield, suitable for downstream applications such as Polymerase Chain Reaction (PCR) and Next-Generation Sequencing (NGS). NGS is a sequencing technology that employs parallel sequencing of numerous small DNA fragments to determine the sequence (Rizzo, 2012; Attia, 2016).

Materials and Methods

Plant material

The collection of red mangroves (*Rhizophora mangle*) and black mangrove (*Avicennia germinans*) leaf samples was facilitated by personnel from UNICACH under Scientific Collection License: SGPA/DGVS/03408/16, as part of the project "Comparative analysis of spatial genomic differentiation along the Mexican Pacific in two mangrove species, *Rhizophora mangle* L. and *Avicennia germinans* (L.) L." A total of 28 samples were obtained for *A. germinans* and 21 samples for *R. mangle*, which were collected from different regions: 1) Chiapas; 2) Oaxaca; 3) Jalisco; 4) Sinaloa; 5) Sonora; 6) Baja California Sur. These samples were combined to achieve sufficient capacity for antioxidant analysis.

Reagents and instrumentation for antioxidant capacity

The chemicals used in this study, such as Folin-Ciocalteu, sodium carbonate, trihydrated sodium acetate, 2,4,6-tripyridyl-s-triazine (TPTZ), iron (III) chloride hexahydrate, catechin, aluminum chloride, potassium acetate, potassium persulfate, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), vanillin, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and gallic acid, are all products from Sigma-Aldrich (St. Louis, MO, USA). To quantify phenols, tannins, flavonoids, and antioxidant capacity, absorbances were measured using a Synergy 2 Microplate Reader with Gen5 software (Bio-Tek Instruments Inc., Winooski, VT, USA).

Preparation of extracts for antioxidant capacity

The plant material was ground into a powder by placing 2 grams of the sample in a 15 mL centrifuge tube. Simultaneously, 10 mL of 80 % methanol was measured and added to the plant material, mixing it at 1500 rpm using a vortex for five minutes . Once homogeneous, the pH was adjusted to three from an initial five pH in most samples. Then, the tubes were placed in a sonication bath for 15 minutes and incubated at 30°C for 30 minutes. Finally, the tubes were centrifuged at 306.84 g (≈ 3000 rpm) for 15 minutes. The supernatant was collected and added to a flask, adjusted to a volume of 10 mL with 80 % methanol. The extracts were stored in amber glass bottles at an average temperature of 4°C.

Evaluation of antioxidant capacity

An in vitro evaluation of the nutraceutical composition was performed by obtaining extracts with 80 % methanol, following the method described by Hernández-Rodríguez et al. (2016), cited by Espejel-Sánchez (2021). The total phenolic content of each extract was determined using the Folin-Ciocalteu method adapted for microplates (Hernández-Rodríguez et al., 2016). A calibration curve was prepared using a stock solution of gallic acid in a linear concentration range of 2.5 to 29.0 µg/mL-1. The results were expressed as milligrams of gallic acid equivalents per gram of sample on a dry weight basis (mg GAE/g db). On the other hand, total flavonoids were quantified in the extracts using the method described by Kubola and Siriamornpun (2011), with a catechin calibration curve in the concentration range of 0.7 to 34.5 M. For tannin quantification, 4 % vanillin was used with the methanolic extracts acidified with hydrochloric acid at 3 pH, and it was compared against a catechin stock solution at an absorbance of 491 nm in the microplate reader (García-Mier et al., 2015).

DNA extraction

Genomic DNA extraction was performed using the commercial DNeasy Plant Mini Kit (69106) with and without modifications due to the tannic and saline nature of the plant material. Additionally, the CTAB (hexadecyltrimethylammonium bromide) protocol with modifications by Cullings (1992) was executed. All leaves were treated with liquid nitrogen (LN) and ground with a porcelain mortar before extraction, and room temperature (RT) was always maintained between 18-20 °C.

Modifications to the commercial protocol of DNeasy Plant Mini Kit (69106)

For the extraction of genomic DNA, the commercial protocol DNeasy Plant Mini Kit (69106) was used with modifications due to the tannic and saline nature of the plant material. Ethanol was added to the required buffers, and the plant material was cut and placed into flat 2 mL microtubes. Subsequently, 700 µL of buffer AP1 was added, and the sample was ground. After a spin to bring down the sample, 4 µL of RNase was added, and it was incubated at 65°C for 50 minutes, inverting the tubes two to three times. Then, 130 µL of buffer P3 was added, mixed, and incubated on ice for 50 minutes.

The samples were centrifuged for 8 minutes at 2014.7 g (≈ 14,000 rpm) at a temperature of 4°C, and the supernatant (approximately 400 µL) was transferred to a QIAshredder mini spin column, which was centrifuged for 2 minutes at 2014.7 g (≈ 14,000 rpm) and 4°C. 400 µL, or the maximum possible amount of the flowing fraction, was transferred to a new collection tube without any precipitate residue.

Next, 1.5 vol. of the above mixture of buffer AW1 was added, mixed twice, and left to incubate overnight at -20°C. 650 µL of the above mixture (including any precipitate that may have formed) was transferred to a DNAeasy mini spin column, centrifuged for 1 minute at 801.6 g (≈8000 rpm) (4°C), discarding the flow-through. It was centrifuged again (1 minute at 801.6 g (≈8000 rpm) rpm, four °C), discarding the flow-through and the collection tube, and the column was placed in a new collection tube.

A total of 500 µL of buffer AW2 was added, centrifuged for 1 minute at 8000 rpm, and the flow-through was discarded. Then, another 500 µL of buffer AW2 was added and centrifuged for 2 minutes at 2014.7 g (≈ 14,000 rpm) at four °C to dry the membrane. The column was transferred to a 1.5 mL Eppendorf tube, ensuring that the column did not encounter what flowed during the previous step, and 50 µL of buffer AE or molecular-grade water was added directly to the membrane. It was incubated for 5 minutes at room temperature and then centrifuged. Finally, 20 µL of buffer AE or molecular-grade water was added to the membrane, centrifuged for 1 minute at 801.6 g (≈8000 rpm), and discarded the flow-through.

Spectrophotometric analysis of DNA

The concentration, purity (A260/A280 ratio), and absorption ratio at 260-280 nm (A260/A230 ratio) were measured using the NanoDropTM 1000 spectrophotometer from Thermo Scientific (Thermo Fischer Scientific, Germany) using one µL of each sample in a range of 220-750 nm. Additionaly, the sample was measured using a Qubit 3.0 fluorometric system (Thermo Fisher Scientific, Germany), normalizing the DNA concentration to 20 ng/µL with a final volume per sample of 55 µL.

Results and Discussion

Antioxidant capacity

The antioxidants produced by different mangrove species have been well characterized in numerous studies using plant extracts from roots, leaves, and bark. The species that shows the highest antioxidant capacity is the red mangrove (*Rhizophora mangle*), with the samples from Jalisco exhibiting the highest activity at 42.68 mg GAE/g compared to 11.74 mg GAE/g in the black mangrove (*Avicennia germinans*) from Baja California Sur with the highest activity for that species (Figure 1). According to the literature, both species have high antioxidant activity, although *R. mangle* is four times higher than *A. germinans*. For example, in a study conducted with twelve aqueous extracts from different mangrove species on the Yucatan coast, polyphenols and flavonoids were confirmed using the Folin-Ciocalteu reagent and aluminum chloride method. As a result, polyphenols, and flavonoids, known to have antioxidant activity due to their redox properties, were found in all analyzed mangrove species, with the highest activity observed in *R. mangle* and *Manilkara zapota* (Rodríguez-García, 2019). In another study, ethanolic extracts from roots, leaves, and bark of *R. mangle* from the Protected Areas System of Guatemala were analyzed using three methods: DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and TEAC (Trolox Equivalent Antioxidant Capacity). The best antioxidant activity was found in leaf extracts, with a total phenolic content of 637.3 ± 31.3 mg/g; flavonoids showed the highest concentrations (110.1 ± 0.6 ppm), also in the leaves, while tannins had the highest concentration in root samples (5.4 ± 0.1 %) (Cruz S. M., 2015). In

Table 1
Phenolic content, flavonoids, tannins, and antioxidant capacity of two mangrove tree species of the Mexican Pacific coast

Species	State	Phenols	FRAP	Flavonoids	ABTS	Tannins
		mg GAE/g^{-1}*db*	µmol TE/g^{-1}*db*	µmol CE/g^{-1}*db*	µmol TE/g^{-1}*db*	µmol GAE/g^{-1}*db*
Rhizophora mangle	Chiapas	23.30 ± 0.01	230.30 ± 0.01	15.63 ± 0.02	496.24	34.86± 0.03
	Oaxaca	41.95 ± 0.02	95.89	16.30 ± 0.03	241.12 ± 0.02	3.17 ± 0.02
	Jalisco	42.68± 0.03	184.38 ± 0.01	17.56 ± 0.02	297.23	32.42 ± 0.01
	Sinaloa	30.92 ± 0.01	151.16 ± 0.01	29.98 ± 0.03	294.52 ± 0.01	22.99 ± 0.01
	Sonora	33.79 ± 0.01	173.73 ± 0.01	17.88 ± 0.01	341.06 ± 0.02	18.82 ± 0.02
	Baja California Sur	34.24 ± 0.02	164.02 ± 0.01	33.59± 0.03	326.45 ± 0.02	13.25 ± 0.01
Avicennia germinans	Chiapas	8.90 ± 0.02	11.57 ± 0.02	13.02 ± 0.02	24.92 ± 0.02	29.03 ± 0.02
	Oaxaca	9.32 ± 0.03	11.43 ± 0.01	12.86 ± 0.01	28.74 ± 0.02	2.50± 0.03
	Jalisco	5.92 ± 0.01	9.31 ± 0.02	15.44 ± 0.02	15.00 ± 0.03	28.52 ± 0.01
	Sinaloa	3.57 ± 0.02	12.54 ± 0.02	9.95 ± 0.01	24.44 ± 0.02	7.63 ± 0.01
	Sonora	9.14 ± 0.01	14.23 ± 0.01	18.34 ± 0.02	27.94 ± 0.02	19.31 ± 0.01
	Baja California Sur	11.74± 0.03	12.82 ± 0.01	16.24 ± 0.01	25.51 ± 0.01	6.41 ± 0.01

addition to phenols, previous studies have shown that tannins obtained from *R. mangle* extracts have antioxidant capacity due to their ability to reduce DPPH, confirming their free radical scavenging activity (Zhang, 2010), as can be seen in the results obtained for FRAP, ABTS, flavonoids, and condensed tannins.

On the other hand, several reports have demonstrated that extracts from the *Avicennia genus*, such as methanol, ethanol, ethyl ether, acetone, hexane, chloroform, benzene, aqueous, and ethyl acetate, contain novel phytochemicals such as alkaloids, terpenoids, steroids, phenols, saponins, flavonoids, tannins, steroids, and glucosides. As a result, the antioxidant potential has been demonstrated in species like *A. officinalis* (fruit and leaf), as well as the free radical scavenging capacity of *A. marina* (fruit and leaf) in ABTS, DPPH, and FRAP (Ferric Reducing Antioxidant Power) assays (Hrudayanath, 2016). Specifically for *A. germinans*, there is little information on its antioxidant capacity, such as the study conducted with three different mangrove species from a natural reserve in Guatemala, where it was found that leaf and root extracts of *Avicennia germinans* exhibited significant antioxidant activity after evaluation with DPPH, FRAP, TPC (Total Phenolic Compounds using Folin-Ciocalteu reagent), and ABTS methods (Cruz, 2019) (Figure 2). Antioxidant agents reduce free radicals through electron transfer and hydrogen atom transfer. The ABTS and FRAP assays allow for the measurement of free radical reduction through the electron transfer mechanism (Ozgen et al., 2006). Therefore, these two methods corroborated the antioxidant capacity in the polar extracts of red and black mangrove leaves. Andrade-Andrade et al. (2008) mentioned the hypothesis that the type and concentration of phytochemicals vary according to the plant tissue and collection site, which is reflected in the gradient of processed samples in the present study with collection points experiencing maximum temperatures of 33°C with 1600 mm of precipitation in Jalisco, compared to the same temperature in Baja California Sur but with precipitation of 88 mm, having a direct effect on the nature and concentration of secondary metabolites.

DNA extraction

According to O'Neill et al. (2011) and Masago et al. (2021), modifying commercial protocols improved DNA extraction yield. Differences were also observed in the readings of NanoDrop and Qubit due to interfering substances in the sample. The NanoDrop instrument operates on the principle of DNA molecules absorbing light at a specific wavelength (260 nm), with more excellent absorption by nitrogenous bases. The ratio of absorbance at 260 nm and 280 nm indicates DNA purity, as it reflects the presence of proteins and other contaminants that absorb more at 280 nm. In contrast, the Qubit system utilizes fluorophores that bind to DNA and emit a fluorescence signal proportional to the DNA concentration. The specific fluorophore used in the Qubit kit binds specifically to the phosphate groups of DNA, resulting in a fluorescence signal indicative of DNA quantity and providing a higher level of purity and stability for genomic analysis (O'Neill et al., 2011; Masago et al., 2021).

The modified DNA extraction kit yielded higher average concentrations in both species when measured with Qubit and NanoDrop. *Avicennia germinans* had an average concentration of 77.52 ng/µL with Qubit and 348.55 ng/µL with NanoDrop. For *Rhizophora mangle*, the average concentrations were 2.38 ng/µL and 4.56 ng/µL, respectively. This trend was consistent across all individuals (O'Neill et al., 2011; Masago et al., 2021). Conversely, the CTAB protocol resulted in a higher concentration in *A. germinans* when quantified using NanoDrop (70.29 ng/

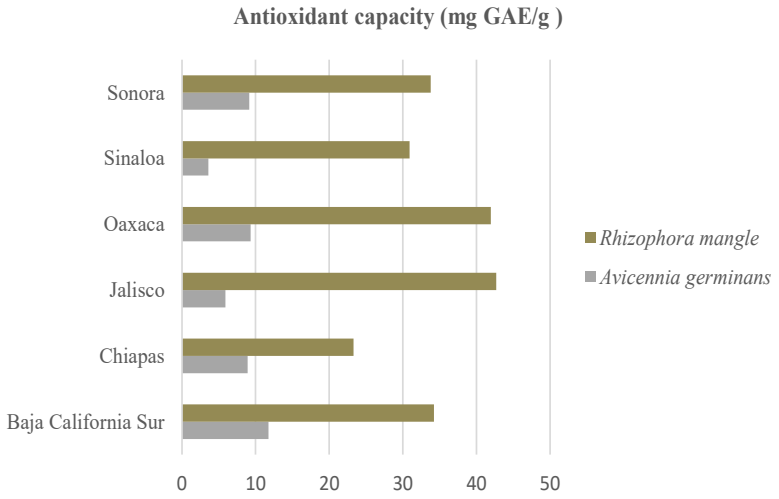


Figure 1
Antioxidant capacity in milligrams of gallic acid equivalents per gram of *Rhizophora mangle* and *Avicennia germinans* sample.

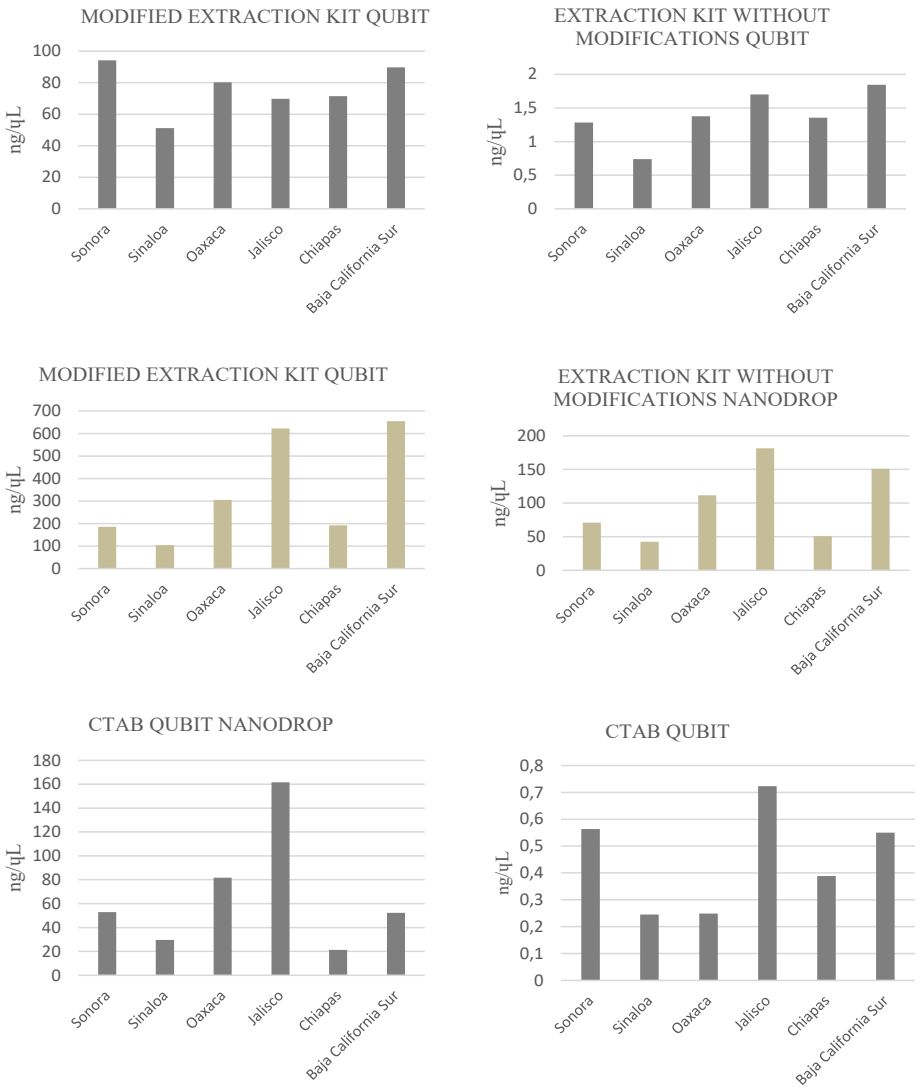


Figure 2
Summary of DNA concentrations per treatment and region

µL) compared to Qubit (0.46 ng/µL). These discrepancies can be attributed to the broader range of absorbances detected by NanoDrop, which includes DNA and other compounds like denatured proteins that remain from the DNA extraction process. In contrast, quantification with a fluorometer provides more accurate results by focusing on the proportional representation of resuspended phosphate groups (O'Neill et al., 2011; Masago et al., 2021).

Conclusions

The results indicated that the modified protocol consistently yielded higher DNA concentrations than the unmodified counterpart. This outcome suggests that the modifications made to the former positively influenced extraction efficiency. Although the CTAB protocol exhibited elevated NanoDrop readings, fluorescence analysis unveiled lower values, possibly indicating genetic material degradation or insufficient purity for genomic endeavors. Furthermore, the choice of quantification method demonstrated its impact on results, manifesting significant divergence in DNA concentrations assessed by the distinct techniques.

Remarkably, in the context of the two mangrove species studied, *Rhizophora mangle* displayed elevated antioxidant activity compared to *Avicennia germinans*. However, intriguingly, the DNA concentration extracted through the *R. mangle* protocol often proved lower than that yielded by the *A. germinans* protocol. This nuanced observation suggests a potential inverse relationship between antioxidant activity and DNA isolation outcomes. This correlation demands deeper exploration to elucidate the complex interplay between antioxidant compounds and the intricacies of DNA extraction processes.

Therefore, as we navigate the intersection of antioxidant capacity and DNA extraction efficacy, our study unveils a compelling avenue for inquiry. The apparent link between these two facets enriches our understanding of these mangrove species and underscores the intricate nature of biological systems and laboratory methods. It raises intriguing questions regarding the potential influence of antioxidants on DNA extraction dynamics, potentially resulting from the complex composition of samples.

In summary, this research provides insights into the multifaceted relationship between antioxidant capacity and DNA extraction results and underscores the necessity of holistic consideration in genomic analyses. Our findings encourage the exploration of biochemical interactions shaping the observed correlation, offering potential refinements in DNA extraction protocols and yielding more profound insights into the biochemical intricacies of these ecologically vital species.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

All data is presented in this manuscript.

Author Contributions (CRedit)

J.M. Cunill-Flores – Conceptualization, Writing an original draft, Methodology.
I. Salgado Escobar – Software, Methodology.
D. Guerra Ramírez – Resources, Methodology.
N. Jiménez-Juárez – Data curation, Investigation.
A. Nettel-Hernanz – Project administration, Methodology, Resources, Funding acquisition.
G.M. Horta-Valerdi – Validation, Supervision.
W.A. Matamoros – Investigation, Supervision.
Y. Pacheco Hernández – Formal analysis, Writing-review & editing. Author Contributions (CRedit):

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