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A Comparison of Four Protocols for the Dna Extraction of Ascomycetes, Genus Xylaria

Viviana Chiluisa-Utreras
Maestría en Biotecnología Molecular y Grupo de Investigación BIOARN, Universidad Politécnica Salesiana, (Ecuador)
Orcid: https://orcid.org/0000-0001-8647-1791

Daniela Álvarez-Guillén
Universidad Politécnica Salesiana, (Ecuador)
Orcid: https://orcid.org/0009-0000-2802-837X

Bryan Parco-Monar
Universidad Politécnica Salesiana, (Ecuador)
Orcid: https://orcid.org/0009-0002-9997-2004

Pamela Loján
Laboratorio de Secuenciamiento de Ácidos Nucleicos, Instituto Nacional de Biodiversidad, (Ecuador)
Orcid: https://orcid.org/0009-0003-7191-8548

Introduction

The genus *Xylaria* belongs to the class Ascomycetes (phylum Ascomycota). These fungi species are characterized by having a compartmentalized mycelium; i.e. septate hyphae can be observed when isolated in a growth medium. Likewise, they have false tissues, known as plectenchyma, which form sexual spores, enabling their sexual reproduction, and conidia, which are asexual spores. Their most distinctive characteristic is the ascus, where the spores reproduce, after karyogamy and meiosis (Salvador, 2019).

The term *Xylaria* derives from the Greek word "xýlon," which means "wood", since these fungi prefer to develop in this natural growth substrate. Species in the genus are characterized by stromas of different dimensions and structure, usually dark. The stems are round and stipitate, with a peltate or pulvinate sheath and several spherical ascomata inside.

As for their ostioles, they are umbilical in shape, and their asci are not continuous; they are round, with an apical pore. Ascospores are found on

the belly in a straight, sigmoid, or spiral shape, and are dark brown or black (Ruiz et al. 2021). It is estimated that there are approximately 400 species of the genus *Xylaria* worldwide; however, new species are frequently discovered in tropical and subtropical regions (Sir et al. 2020).

Species in *Xylaria* have a significant economic importance because they contribute to the carbon cycle through the decomposition of wood. It produces enzymes such as hydrolases or laccases for degrading wood material (Sandoval et al. 2017).

Research on fungal diversity has historically been biased towards temperate regions, leaving tropical areas relatively unexplored (Hawksworth & Lücking, 2017). This geographical bias stems from various factors, including limited access to remote areas, logistical challenges, and a historical focus on well-studied regions (Díaz et al. 2021). As a result, many species in Neotropics remain undiscovered and undescribed, particularly in remote and inaccessible areas (Haelewaters et al. 2025).

Compounding this geographical bias is a lack of taxonomic expertise, particularly in the Neotropics. Identifying and classifying fungi requires specialized knowledge and skills, which are often limited in this region. This scarcity of experts hampers efforts to accurately document and describe fungal species, leading to an underestimation of true diversity and hindering our understanding of the evolutionary relationships among fungal taxa. Commercial kits, which are expensive and most are not accessible or easy to obtain (Papalini et al. 2023), could often represent significant obstacles for developing research in Latin America; thus, in-house extraction methods could represent important alternatives.

One possible way to contribute to closing the knowledge gap in fungal diversity is access to DNA sequences that can inform on the phylogenetic characteristics of fungi. To contribute new knowledge on laboratory techniques for fungi DNA extraction, we compared three in-house DNA extraction protocols and a commercial kit protocol. We present new insights into efficient alternatives for obtaining genomic DNA in fungi samples.

Materials And Methods

Plant material: Herbarium samples were obtained from the National Herbarium of Ecuador (QCNE) of the National Institute of Biodiversity (INABIO). The absence of pathogens was considered, and especially, the presence of a fruiting body pulvinar, as this indicates the presence of spores in the structure (Batallas, 2020). Likewise, fresh samples were collected from the orchid park "El Pahuma," located in the parish of Nanegalito approximately 45 km northwest of Quito, with the following coordinates 0.064°S and 78.63°W, using the authorization number provided by the Ministry of the Environment under

contract number MAATE Code – AR-SFC – 2023 – 3422.

Experimental design: For the statistical analysis, a completely randomized experimental design ANOVA was used in a 4x3 factorial analysis, i.e., 3 protocols + 1 commercial kit, resulting in 4 protocols to evaluate and 3 states of the sample (young tissue and preserved hydrated and non-hydrated), resulting in 12 treatments and 4 repetitions applied for each protocol, with a total of 48 experimental units. A *post hoc* Tukey test was used to evaluate the effect of the DNA extraction protocols on the samples, determining which protocol is statistically significant and therefore effective or at least distinct in its effect. In this case, the variable evaluated was the presence of DNA extracted from the sample, which will be quantified in units such as ng/µL.

Tissue classification

The collected samples were classified into three categories: young sample (recently collected), old sample (preserved in herbarium), and hydrated sample, which was divided into two types: non-hydrated and hydrated and labeled as follows: MJ (young sample), MV-NH (non-hydrated old sample), and MV-H (hydrated old sample). For the latter, a 10% glucose solution was used for hydration. The three kinds of tissue were cut longitudinally. The tissues were then placed in 1.5 mL Eppendorf tubes, labeled, and stored in an ultra-freezer (-80 °C) to contribute to cell lysis; this process took place between one and four weeks.

Molecular analysis

Protocol 1

We modified the method mentioned by Laurentín & Martínez-Hilders (2012). We used 120 µL of the lysis buffer, consisting of 2% CTAB, mercaptoethanol (1M), and proteinase K (0.714 mM). To denature cell proteins, we used a solution consisting of phenol, chloroform, and isoamyl alcohol in a 25:24:1 ratio. The resulting suspension was centrifuged for 11 min at 13,300 rpm; then, we added 750 µL of cold absolute ethanol and 5 µL of ammonium acetate (2.5 M). The samples were incubated at -20 °C for 20 minutes. The resulting pellets were centrifuged and preserved. The pellets were washed twice with 200 µL of cold 80% ethanol and allowed to dry for 1 hour at room temperature, with the tube lids open. To dilute the genetic material, we added 50 µL of TE buffer. Finally, we extracted 20 µL of the suspension and incubated it with 1.25 µL of RNase for 1 hour at 37 °C. The genetic material obtained was stored at -20 °C.

Protocol 2

We followed the method described by Huanca et al. (2014) with modifications. The tissues stored in the ultra-freezer were placed in a 500 µL lysis solution composed of: 2% CTAB; TrisHCl (100 mM); NaCl (2.4M); 5% DMSO; EDTA (20 mM) and lithium chloride (1M), with a pH adjusted to 8. We prepared a second solution consisting of chloroform and isoamyl alcohol

in a ratio of 24:1. The prepared tissues were centrifuged at 12,000 rpm for 10 minutes. The supernatant was removed, and cold absolute ethanol was added dropwise. The samples were then stored at −20 °C for 30 minutes. Subsequently, the samples were centrifuged at 13,300 rpm for 11 minutes, and 500 µL of 70% ethanol was added. Samples were then centrifuged at 12,000 rpm for 5 minutes. Finally, the pellets were dried at room temperature and dissolved with 50 µL of ultrapure water and stored at –20 °C.

Protocol 3

We considered the method by Gómez et al. (2019) with modifications. The three types of tissues that were in the ultra-freezer were placed in 500 µl of a lysis solution containing Tris-HCl (200 mM), NaCl (250 mM), EDTA (2.5 mM), and 0.5% SDS. Subsequently, it was centrifuged at 10,000 rpm for 5 minutes, and 150 µL of cold chloroform was added and centrifuged at 13,000 rpm for 15 minutes. One volume of chloroform was added and centrifuged at 13,000 rpm for 10 minutes. 500 µL of cold ethanol was added and centrifuged for 5 minutes. An aliquot of 500 µl of cold 70% ethanol was added, and the pellet was resuspended with fingers. Afterwards, it was centrifuged at 13,000 rpm for 2 minutes, then 500 µL of cold 70% ethanol was added again and centrifuged for 1 minute. Finally, the pellet was dried at room temperature for approximately 30 minutes, suspended in 50 µL of ultrapure water, and stored at -20 °C for further analysis.

Protocol 4

We used the Gene JET plant genomic DNA purification kit (Thermo Scientific). Tissues were submerged in 350 µL of lysis buffer A. Then, 50 µL of lysis buffer B and 10 µL of RNase A were added. It was then incubated for 10 minutes at 65 °C with shaking. 130 µL of protein precipitation solution was added. It was then incubated on ice for 5 minutes and centrifuged for 6 minutes at 13,300 rpm. The supernatant was collected, and 400 µL of Binding Solution and 400 µL of 96% ethanol were added. This mixture was transferred in two parts to a sterile column and centrifuged for 1 minute at 8000 rpm, this process was repeated twice. An aliquot of 500 µL of wash buffer was added and centrifuged for 1 minute at 10,000 rpm, and 500 µL of wash buffer II was added and centrifuged at 13,300 rpm for 4 minutes. It was then centrifuged for 1 minute. Finally, 75 µL of elution buffer was placed on the column and incubated for 5 minutes at room temperature, then centrifuged for 1 minute at 10,000 rpm, and this step was repeated to obtain purified DNA. The sample was stored at -20 °C.

Quantification of extracted DNA

The concentration and purity of the extracted DNA were assessed using a Nanodrop spectrophotometer. For Protocol 1, Tris EDTA (TE) buffer was used as a blank. For Protocols 2 and 3, molecular-grade water was used as a blank. As for the commercial kit, the elution buffer was used as a blank. The spectrophoto-

meter was reset with blanks every five readings. After quantification, concentration and absorbance measurements at 260/280 nm and 260/230 nm were recorded for each tissue type analyzed.

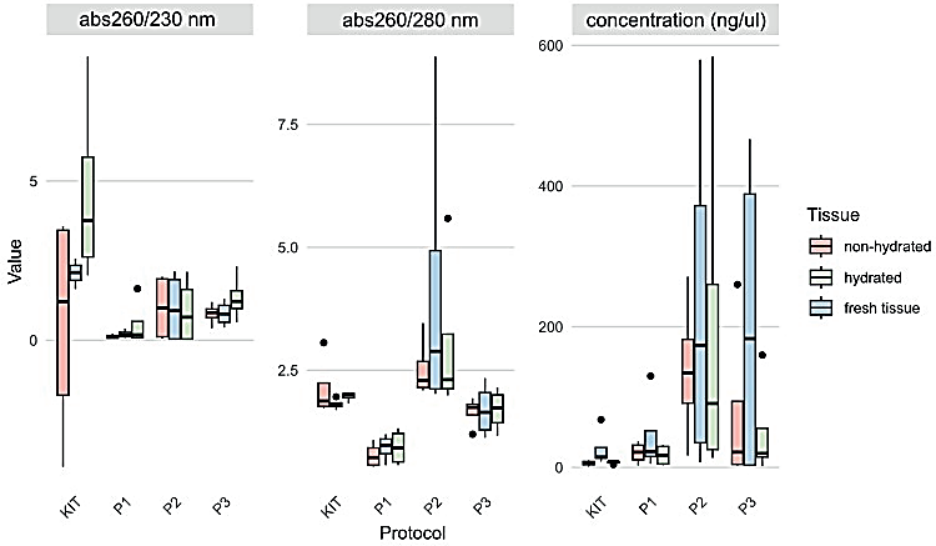
Statistical analysis

We used a completely randomized ANOVA experimental design in 4×3 factorial analysis; i.e. 3 protocols + 1 commercial kit, resulting in 4 protocols to be evaluated and 3 sample states (MJ, MV-NH, and MV-H). The resulting 12 treatments were measured through 4 replicates, for a total of 48 experimental units. We used a Tukey *post hoc* test to determine which specific extraction protocol and sample was statistically significant. Previous to the ANOVA, we applied a Shapiro-Wilks test to assess normality in the obtained data. Thus, to test the effect of each main treatment (concentration in ng/ml, 260/280 absorbance in nm, and 260/230 absorbance in nm), we performed three ANOVA tests, each with the type of tissue as a control factor.

Results

DNA concentration (F=4.06, df=3, P=0.014), 260/280 absorbance (F=9.34, df=3, P<0.001), and 260/230 absorbance (F=4.13, df=3, P=0.0129) were all statistically significant in the ANOVA test; tissue sample was not a significant factor overall (Figure 1).

Figure. 1. *Box plot for the results of the experiment in the assessment of four DNA extraction protocols. Protocol 1 (P1); Protocol 2 (P2); Protocol 3 (P3) and commercial kit (KIT)"*



Contrasts for DNA concentration

In terms of performance, the individual protocols evaluated showed significant differences in both the purity and concentration of extracted DNA. According to the Tukey test, protocol 2 was statistically different from Protocol 1 and the commercial kit, but not from Protocol 3 (Error = 17681.1, gl = 42, P<0.05) and also presented the highest DNA concentration ($\bar{x}$ = 189.18, SE = 38.39), reaching values that in some cases exceeded 300 ng/µL, especially in fresh tissue (Figure 1).

Notably, the use of the commercial kit resulted in low concentration of DNA in contrast to what was established in the reference product manual (Figure 1). The difference between obtained and expected results can be attributed to the specificity of the extraction kit for plants instead of fungi. However, the purity values obtained with the commercial kit were optimal ($\bar{x}$ = 13.26, SE = 38.39) (Figure 1), within the appropriate range to ensure DNA integrity, a result favored by silica columns that facilitate a more efficient elimination of contaminants.

Contrasts for the 260/280 and 260/230nm ratios

After the individual contrasts provided by the Tukey test, the commercial kit was significantly different from the other three protocols for the 260/280 nm (Error = 1.1828, gl = 42, P<0.05). The individual differences for the 260/230 nm were more complex, with the commercial kit, Protocol 3 and 2 forming one cluster, and Protocol 1, Protocol 2 and Protocol 3 forming another cluster; the statistically significant difference in this case being between the commercial kit and Protocol 1 (Error = 17681.1, gl = 42, P<0.05). When analyzing DNA purity, absorbance ratios at 260/280 nm and 260/230 nm were lower for the commercial kit, which means better values. Specifically, the 260/280 nm absorbance ($\bar{x}$= 1.97, SD= 0.31) and 260/230 nm absorbance ($\bar{x}$= 2.41, SD= 0.46) for the commercial kit were within the ideal expected ranges (1.8-2.0 and 2.0-2.2, respectively) (Figure 1). In addition, for the commercial kit, there was less variance dispersion when compared to the other protocols (Figure 1). This lower variability for the commercial kit suggests greater consistency between replicates. The other protocols, although in some cases achieving higher purity values, showed greater variability between tissue types.

Discussion

Contrasts for DNA concentration

According to the results highlighted in Figure 1, Ríos et al. (2016) used the same organic solvents as in Protocol 2 but obtained a concentration of 136.76 ng/µL and a purity of 1.71 µL, like the highest values reached in our research. Similarly, Dilhari et al. (2017) indicated

that it is important to maintain DNA in alkaline conditions, as this prevents base depurination; a pH of 8 is the most appropriate. The temperature and incubation time of the sample, as well as the drying techniques, can alter the concentration or purity of the extracted DNA sample.

Contrasts for the 260/280 and 260/230nm ratios

These results indicate that, while Protocol 2 maximizes the amount of recovered DNA, the commercial kit provides optimal purity, which may be more suitable for applications that require contaminant-free DNA, such as PCR or sequencing. Therefore, the choice of protocol will depend on the required balance between concentration and purity, as well as the type of samples used. According to Gand et al. (2023), DNA purity is a critical factor that can influence the success of PCR reactions. It was emphasized that contaminants such as proteins or solvent residues can inhibit PCR, and that extraction methods that produce DNA with A260/280 ratios between 1.7 and 2.1 are more suitable for molecular applications. Therefore, while Protocol 2 maximizes the amount of DNA recovered, the commercial kit offers more uniform quality, which may be more convenient for sensitive molecular applications.

Conclusions

Protocol 2 stands out as the most effective in terms of concentration of the extracted genetic material. The commercial kit had better performance to remove organic solvents and proteins. A complementary approach that takes advantage of the combined benefits provided by Protocol 2 and the commercial kit is recommended to obtain DNA of both high purity and integrity. Further validation is required to test the efficacy of a hybrid protocol including the advantages of both Protocol 2 and the commercial kit. The selection of the most appropriate protocol will depend on the required balance between concentration, purity and the final purpose of the extracted DNA.

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