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Liquid-nitrogen-free CTAB DNA extraction method from silica-dried specimens for next-generation sequencing and assembly ☆

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ABSTRACT

Next-generation sequencing requires intact and high-quality DNA. However, typical liquid-nitrogen DNA extraction methods are expensive and not practical for field sample collections. Hence, we present a cost-effective method for DNA extraction from silica-dried leaf samples, eliminating the need for liquid nitrogen. Two protocols were evaluated to determine the effectiveness of grinding dried plant samples without liquid nitrogen in comparison to the standard protocol for tissue homogenization and cell lysis. Protocol 1 involved grinding fresh leaf samples with liquid nitrogen, while Protocol 2 entailed incubating dried plant samples at-20 ˚C for 1 h before grinding in the absence of liquid nitrogen. Both protocols produced comparable DNA yields with an average A260/A280 ratio of 1.78±0.02, suitable for short- and long-read sequencing.

☆ **Related research article:** R.P. Jr.Gentallan, S. Sengun, M.C. Bartolome, K.J.O. Quiñones, N.B. Coronado, T.B. Borromeo, E. B.S. Timog. The *Vitex trifolia* complex (Lamiaceae) in the Philippines. PhytoKeys (2024) (*in press*)

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Using Protocol 2, we successfully assembled ten plastomes. It also demonstrated versatility as comparable DNA quality was obtained from dried mollusks and actinomycetes, resulting in the successful assembly of two complete mitochondrial genomes. The protocol is advantageous for research workflows involving the collection of samples in the field as a long-term source of genetic material.

- Drying: Fresh samples were silica-dried at silica-to-sample ratio of 2:1.
- Pre-lysis: Dried samples were frozen at −20 °C for 1 hour before grinding.
- Frozen samples were subjected to tissue homogenization followed by the standard CTAB DNA extraction.

Specifications table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	*Plant genomics, Bioinformatics*
Name of your method:	Liquid-Nitrogen-Free CTAB DNA Extraction Method
Name and reference of original method:	[1]. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical bulletin.
Resource availability:	GitHub - Kinggerm/GetOrganelle: Organelle Genome Assembly Toolkit (Chloroplast/Mitocondrial/ITS); CPGAVAS2; MPI-MP CHLOROBOX - OGDRAW (mpg.de)

Method details

Background

The cetyltrimethylammonium bromide (CTAB) method, popularized by Doyle and Doyle [1], has been widely used for DNA extraction. Due to the plant's unique physical and chemical characteristics and properties, CTAB method has undergone numerous modifications primarily tailored to different plant groups. One of the modifications in the protocol for optimization is in tissue homogenization and cell lysis [2], a crucial step where DNA is released from the cells and oxidation of phenolic compounds occurs [3]. Fresh plant tissue samples are typically homogenized and lysed in the presence of liquid nitrogen. This helps deactivate harmful enzymes and chemicals, preventing DNA degradation. However, the use of liquid nitrogen is expensive, and it is often unavailable in remote and less-equipped laboratories, thereby hindering DNA extraction. Adopting a method that eliminates the need for liquid nitrogen and involves few reagents would present a practical and cost-effective alternative.

On the other hand, desiccants such as silica gels have been used for preserving plant tissues and demonstrated as almost comparable source and optimal for DNA extraction [4,5]. This cost-effective and straightforward technique rapidly halts plant cellular activities prior to undergoing apoptosis-triggered DNA hydrolysis, and it prevents the accumulation of oxidative polyphenols during senescence and wound response [6]. Hence, in this study, we present a modified protocol for DNA extraction using silica-dried plant samples without the use of liquid nitrogen. The modified protocol was compared to another protocol that involved the use of liquid nitrogen, aiming to assess both the quality and quantity of the extracted DNA. The newly developed protocol offers a cost-effective approach by (1) eliminating liquid nitrogen, (2) reducing the number of reagents, and (3) being suitable for silica-dried samples. Two (2) protocols were employed for DNA extraction, with variations in sample types (e.g., fresh and silica-dried) and the use of liquid nitrogen for tissue homogenization and cell lysis, in order to assess the integrity of the extracted genomic DNA. All modified methods were based on the CTAB method by Doyle and Doyle [1].

Collection of plant material

Fresh, healthy and young to mature leaves (2–3) were collected and dried following the modified method of Chase and Hills [4]. The leaves were pat-dried with a clean tissue to remove dirt and other debris before placing it in a clean coffee filter. This was sealed with masking tape to avoid direct contact with the silica gels.

Reagents and solutions

The extraction buffer was composed of 2 % Cetyltrimethylammonium bromide, 1.4 M NaCl, 0.1 M Tris–HCl, 0.02 M EDTA (pH 8), 0.2 % β-mercapto-ethanol, 2 % PVP, chloroform:isoamyl (24:1), iced-cold absolute isopropyl alcohol, 70 % ethanol, 1X TE buffer and TBE (Tris, Boric acid, EDTA). All the materials used in the study were first sterilised and autoclaved.

DNA isolation protocol

The modified protocols were first implemented on the different accessions of *Vitex* spp., the interest of our research program.

Protocol 1:

1. Pre-cool mortar and pestle by adding a small amount of liquid N_2.
2. Grind 0.5 g plant sample into the pre-cooled mortar and pestle then transfer the powder into the pre-heated 15-mL centrifuge tube with CTAB buffer (4 mL). Make sure to resuspend the powdered sample into the buffer.

3. Incubate the sample for 1 h at 63 ˚C. Swirl the tube every 15 mins.
4. Transfer 900 μL of the incubated sample into a clean sterile 2.0-ml microcentrifuge tube (for 4 mL CTAB buffer (you can have 3 (900 μL) microcentrifuge tubes per sample which can serve as replicates).
5. Add an equal volume (900 μL) of chloroform:isoamyl (24:1). Mix the solution by inverting the tubes (20–30 times) vigorously. Release the air by opening the cap.
6. Centrifuge for 10 mins at 10,000 rpm at room temperature.
7. Transfer 700 μL supernatant (aqueous solution) to a clean sterile microcentrifuge tube. Do not include the interphase part of the solution.
8. Add an equal volume (700 μL) of absolute ice-cold isopropanol. Mix the solution by carefully inverting the tubes 3–4 times.
9. Incubate overnight at −20 ˚C.
10. Centrifuge for 10 mins at 10,000 rpm at room temperature. Decant the supernatant carefully.
11. Wash pellets by adding 1 mL 70 % ethanol. Invert 2–3 times carefully.
12. Centrifuge at 10,000 rpm at room temperature for 10 mins. Decant the supernatant.
13. Repeat steps 11 and 12.
14. Air dry pellet for 10–15 mins.
15. Resuspend the pellet with 1X TE buffer.
16. Add RNase. Pipette up and down to mix thoroughly.
17. Incubate at 37 ˚C for 1 h.
18. Check the quality and quantity using a spectrophotometer (DeNovix® DS-11+) and visualize it through gel electrophoresis.
19. Store at −20 ˚C.

Protocol 2

Protocol 1 was further modified by using small amounts (30–50 mg) of silica-dried sample tissues pulverized in tissue lyser (TissueLyser II Qiagen®) without the use of liquid nitrogen (Protocol 2).

1. Prepare 2.0 mL microcentrifuge tubes for tissue and cell lysis. Add 3 pieces of sterile grinding beads (tungsten carbide or metal beads) into the tubes.
2. Insert the 2.0 mL-microcentrifuge tube with the grinding beads into the tissue lyser adapter.
3. Pre-cool the tubes at −20 ˚C.
4. Weigh 30–50 mg of the silica-dried leaf samples. (Use sterile scissors when cutting the leaves)
5. Place the silica-dried samples (30–50 mg) into the pre-cooled 2.0 mL-microcentrifuge tube with grinding beads.
6. Incubate the samples at −20 ˚C for 1 h.
7. Pre-heat the CTAB buffer at 63 ˚C.
8. Using the tissue lyser grinder, homogenize the silica-dried sample at 45 Hz for 30 s.
9. Remove tubes from the adapter, then add 1 mL CTAB buffer. Make sure to resuspend the powdered sample into the buffer.
10. Incubate the sample for 1 h at 63 ˚C. Swirl or mix the samples every 15 mins.
11. Transfer 900 μL of the incubated sample into a clean sterile 2.0-ml microcentrifuge tube. Do not include the beads.
12. Add an equal volume (900 μL) of chloroform:isoamyl (24:1). Mix the solution by inverting the tubes (20–30 times) vigorously. Release the air by opening the cap.
13. Centrifuge for 10 mins at 10,000 rpm at room temperature.
14. Transfer the 700-μL supernatant (aqueous solution) to a clean sterile microcentrifuge tube. Do not include the interphase part of the solution.
15. Add equal volume (700 μL) of absolute ice-cold isopropanol. Mix the solution by carefully inverting the tubes 3 – 4 times.
16. Incubate overnight at −20 ˚C.
17. Centrifuge for 10 mins at 10,000 rpm at room temperature. Decant the supernatant carefully.
18. Wash pellets by adding 1 mL 70 % ethanol. Invert 2 – 3 times carefully.
19. Centrifuge at 10,000 rpm at room temperature for 10 mins. Decant the supernatant.
20. Repeat steps 18 and 19.
21. Air dry pellet for 10 – 15 mins.
22. Resuspend the pellet with 1X TE buffer.
23. Add RNase. Pipette up and down to mix thoroughly.
24. Incubate at 37 ˚C for 1 h.
25. Check the quality and quantity using a spectrophotometer (DeNovix® DS-11+) and visualize it through gel electrophoresis.
26. Store at −20 ˚C.

Protocol assessment and evaluation

The extracted genomic DNAs were evaluated through gel electrophoresis using 1 % agarose gel in a Tris-Borate-EDTA (TBE) solution. The extracted DNA using Protocols 1 and 2 from the different accessions of *V. trifolia* and *V. negundo* produced an intact DNA band in the gel (Fig. 1). Using small amounts of plant samples (30–50 mg), considerable amounts of DNA was extracted using Protocol 2 with an average purity of 1.8 ± 0.05 (Table 1).

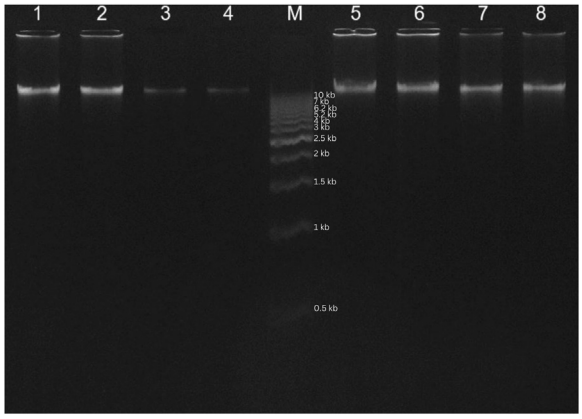

Fig. 1. Genomic DNA from different accessions of *V. negundo* and *V. trifolia* using the modified CTAB protocols. Lanes 1–4 correspond to the extracted DNAs from *V. negundo* accessions, while Lanes 5–8 correspond to *V. trifolia*. Specifically, Lanes 1, 2, 5, and 6 extracted DNA using Protocol 1 (fresh samples ground in liquid nitrogen) while Lanes 3, 4, 7, and 8 used Protocol 2 (pulverized silica-dried samples without using liquid nitrogen).

Table 1
List of species from which DNA was extracted for sequencing, and chloroplast assembly.

Division	Family	Sample	Protocol used	Yield* (ng/μL)	A260/280	Q20 (%)	No. of circularised genomes
Angiosperm/dicot	Lamiaceae	*Vitex negundo*	1	41.40	1.893	96.91	2
			2	49.00	1.739	97.03	2
		Vitex trifolia	1	95.20	1.921	97.30	1
			2	27.20	1.739	97.03	1

* using fluorometer.

Table 2
Quantity and quality of the extracted DNA from various organisms using Protocol 2.

Division	Family	Sample	Yield (ng/μL)	A260/280	No. of circularized genomes
Gymnosperm	Cycadaceae	*Cycas* sp.	495.97	1.77	–
	Gnetaceae	*Gnetum gnemon*	858.29	1.87	–
Angiosperm/Monocot	Nymphaeaceae	*Nymphaea* sp.	947.23	1.74	–
	Orchidaceae	*Vanilla* sp.	165.24	1.48	–
	Poaceae	*Oryza officinalis*	440.46	1.72	1
		Oryza rufipogon	451.35	1.75	1
		Oryza meyeriana	413.70	1.72	–
		Oryza minuta	241.07	1.73	–
		Zea mays	2630.11	1.92	–
		Sorghum sp.	638.19	1.91	–
	Zingiberaceae	*Curcuma* sp.	90.42	1.73	2
		Hedychium sp.	369.47	1.85	2
Angiosperm/Dicot	Annonaceae	*Cananga odorata*	849.43	1.78	–
	Convolvulaceae	*Ipomea* sp.	1545.61	1.95	–
	Lamiaceae	*Tectona grandis*	173.22	1.75	–
		Patraeovitex sp.	1276.69	1.79	–
		Vitex parviflora	118.904	1.47	1
Actinobacteria	Streptomycetaceae	*Streptomyces* sp. KSUA01	486.07	1.68	–
		Streptomyces sp. KSUA22	775.00	1.77	–
Mollusk	Mytilidae	*Mytella strigata* M001	1577.83	1.87	1
		Mytella strigata M002	1996.29	1.85	1

Assembly of chloroplast genome

To assess the efficacy and quality of DNA extracted using both protocols, genomic DNA (gDNA) (50–100 μL) from representative accessions of the two species used in Protocols 1 and 2 were sent to NovogeneAIT Genomics Singapore PTE LTD, Singapore, for sequencing using the HiSeq-PE150 platform (Illumina Inc., San Diego, CA, USA), which was then used for chloroplast assembly. This was done to determine and confirm whether the extracted DNA from Protocols 1 and 2 will yield short-read sequences that can be subsequently assembled into identical chloroplast genomes.

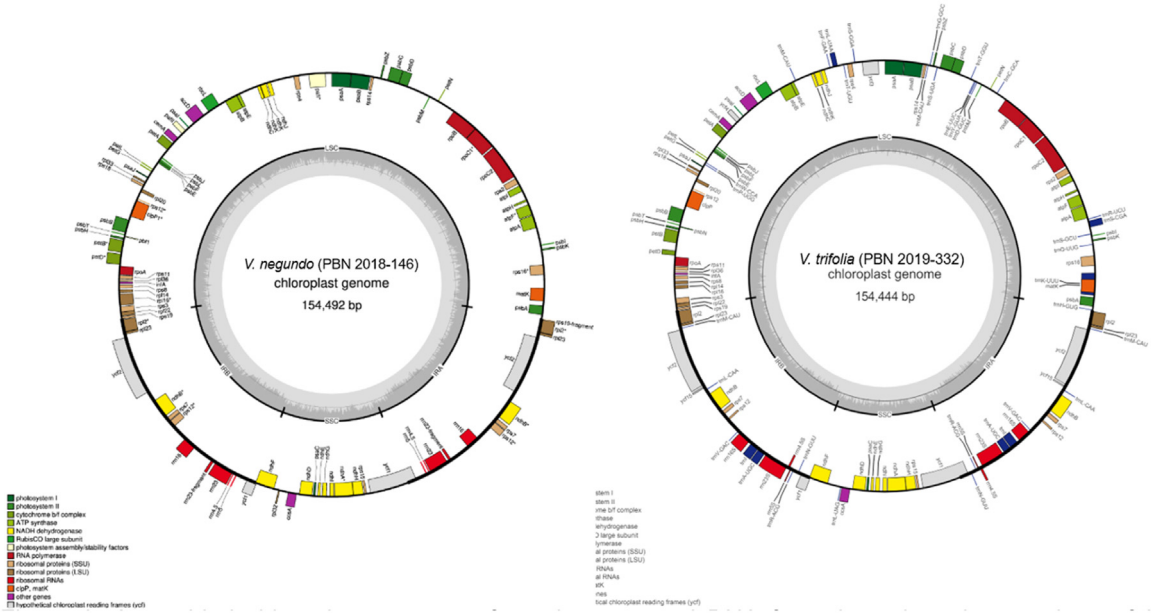

Fig. 2. Assembled chloroplast genomes from the extracted DNA from the selected accessions of *V. negundo* and *V. trifolia* using Protocol 2. Genes shown inside of the circle indicate a clockwise transcriptional direction, while those shown outside are in a counterclockwise direction. Genes belonging to different functional categories are indicated in different colors.

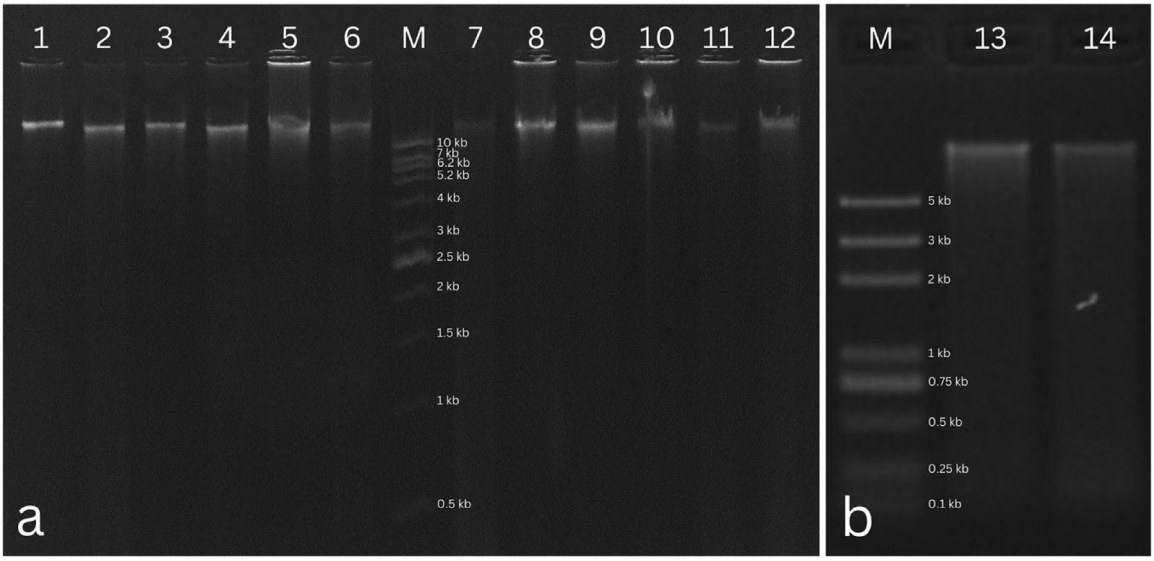

Fig. 3. Agarose gel (1 %) electrophoresis of some of the extracted genomic DNA from different organisms using Protocol 2. (a) Lane 1 – *O. officinalis*, 2 – *O. rufipogon*, 3 – *Z. mays*, 4 – *Sorghum* sp., 5 – *Nymphaeae* sp., 6 – *C. odorata*, 7 – *Vanilla* sp., 8 – *Ipomea* sp., 9 – *Cycas* sp., 10 – *G. gnemon*, 11 and 12 – *Streptomyces* sp. KSUA01 and *Streptomyces* sp. KSUA22, M – DNA Ladder (1 kb), (b) M – DNA ladder (5 kb), 13 and 14 – *M. strigata* accessions (M001 and M002).

The extracted DNA from the two protocols successfully produced 150-bp paired-end raw reads which were successively filtered to cleaned reads then assembled into circular genomes using GetOrganelle v1.7.5+ software [7]. Using CPGAVAS2 [8], the complete chloroplast genomes of *V. negundo* and *V. trifolia* accessions were assembled into a circular genome and were visualised using OGDRAW [9]. The assembled chloroplast genomes from the extracted DNA using Protocols 1 and 2 from *Vitex* spp. exhibited the same genomic features. They possessed the typical quadripartite structure and inverted repeats separating the small and large copy regions (Fig. 2). Protocol 1, which utilizes fresh leaves ground in liquid nitrogen, was used for extracting DNA from *V. trifolia* [10], *Citrus micrantha* [11], *V. parviflora* [12], *Mentha* × *villosa* [13], *V. bicolor* [14], *Senna alata* [15] and *Allium chinense* [16]. The assembled chloroplast genomes are used as plant super-barcodes to elucidate the evolutionary relationship of closely related species [14].

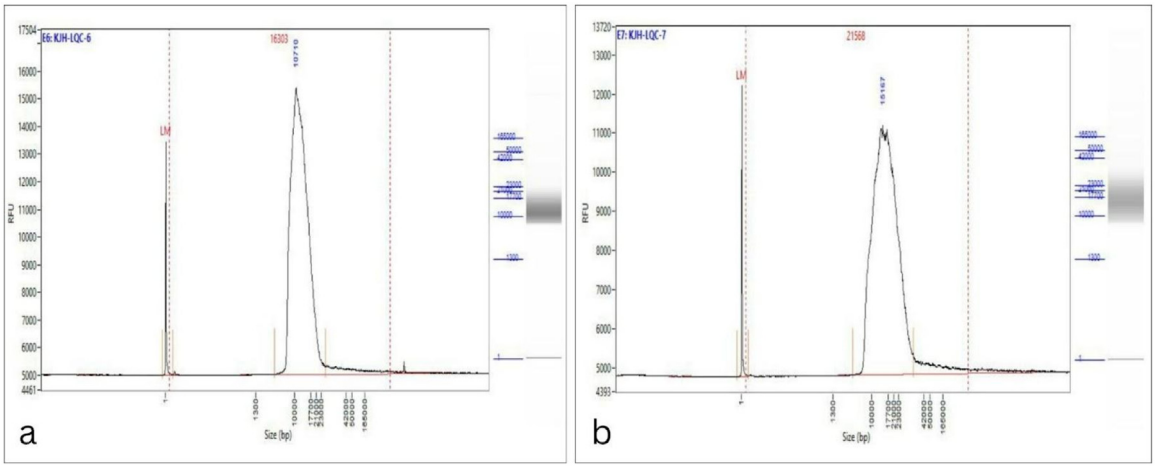

Fig. 4. Quality and size of chromatogram peaks obtained from the library of extracted genomic DNA from (a) *O. rufipogon* and (b) *O. officinalis*, intended for utilization in long-read sequencing.

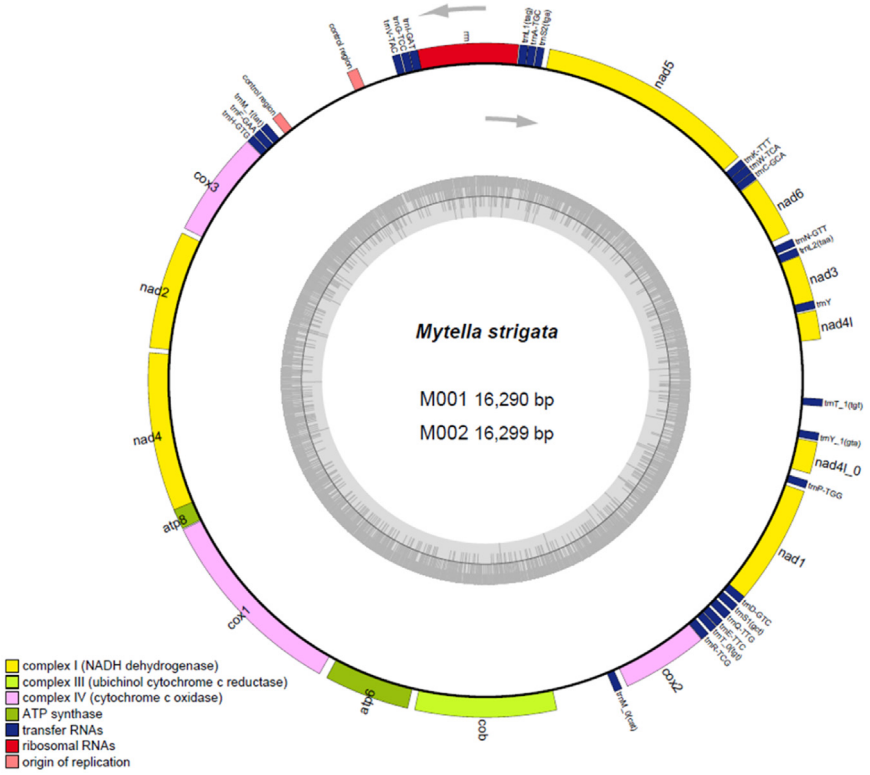

Fig. 5. Gene map of the *Mytella strigata* (M001 and M002) mitochondrial genomes. Genes shown inside of the circle indicate a clockwise transcriptional direction, while those shown outside are in counterclockwise direction. Genes belonging to different functional categories are indicated in different colours.

DNA isolation using protocol 2 for other species

We further evaluated the protocol that involves silica-dried samples, ground in TissueLyser II without using liquid nitrogen (Protocol 2), to evaluate its adaptability in various species including bacteria and mollusk accessions (Table 2). Using a spectrophotometer (DeNovix® DS-11+), the purity and quantity of the extracted DNAs were measured through its A260/280 values. Protocol 2 yielded an intact and large quantity (90–860 ng/μL) with an average DNA purity of 1.77±0.03 from different taxa, such as from gymnosperms (*Gnetum gnemon* and *Cycas* sp.) and other angiosperms (monocot/dicot) species (Table 2, Fig. 3). The extracted DNAs from *Oryza ru-*

fipogon and *O. officinalis*, representatives of the monocot group, were sent for long-read sequencing (PacBio HiFi), resulting in library sizes of 16,303 and 21,568 bp, respectively, which suffice the requirement size for whole genome sequencing (Fig. 4).

For bacteria and mollusks, *Streptomyces* sp. and *Mytella strigata* were used, respectively. For drying, *Streptomyces* sp. was initially harvested from its media and transferred into sterile 2-mL microcentrifuge tubes. Meanwhile, the fresh mussel foot of the mollusk was submerged in 70 % ethanol. These samples were dried by placing them in large bottles with silica gels, with the tubes left open until they were fully dried. Using Protocol 2, the DNA from bacteria and mollusks were successfully extracted (Fig. 2). The extracted DNA from *M. strigata* was sequenced using the Illumina platform (HiSeqPE 150), which generated a sufficient number of raw reads. These were then successfully assembled using GetOrganelle v1.7.5+ software [7], generating a circular genome that was subsequently annotated and mapped using CPGAVAS2 [8] and visualized using OGDRAW [9] (Fig. 5).

Although smearing in the gel is present, indicating DNA degradation, the extracted DNAs were still able to produce the required quality and amount for sequencing to proceed. This confirms that Protocol 2, which utilizes small amounts of silica-dried samples, demonstrates the ability to extract DNA across various taxa. Consequently, it is suitable for both short and long-read sequencing for chloroplast/mitochondrial and whole-genome analyses. This method is especially advantageous for research procedures that require sample collection in the field as a long-term source of genetic material and offers a practical solution for resource-limited laboratories. However, further optimization of the protocol for specific organisms is recommended, and exploring the use of an indigenous bead-based lyser could be beneficial towards achieving cost-effectivity, particularly in resource-limited laboratories.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Kristine Joyce O. Quiñones: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Renerio P. Gentallan Jr:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Funding acquisition. **Emmanuel Bonifacio S. Timog:** Validation, Visualization, Writing – review & editing. **Juan Rodrigo A. Vera Cruz:** Validation, Visualization, Writing – review & editing. **Cherray Gabrielle A. Macabecha:** Validation, Visualization, Writing – review & editing. **Irene A. Papa:** Conceptualization, Supervision, Writing – review & editing. **Nadine B. Coronado:** Validation, Visualization, Writing – review & editing. **Michael Cedric B. Bartolome:** Validation, Visualization, Writing – review & editing. **Daryl B. Ceribo:** Validation, Visualization, Writing – review & editing. **Roselle E. Madayag:** Validation, Visualization, Writing – review & editing. **Jessabel B. Magtoltol:** Validation, Visualization, Writing – review & editing. **Robert Keith A. Sienes:** Validation, Visualization, Writing – review & editing. **Bartimeus Buiene S. Alvaran:** Validation, Visualization, Writing – review & editing. **Teresita H. Borromeo:** Conceptualization, Supervision, Writing – review & editing.

Data availability

Data will be made available on request.

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