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EVALUATION OF GENOMIC DNA EXTRACTION PROTOCOLS IN VANDACEOUS ORCHIDS: A COMPARATIVE APPROACH

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ABSTRACT

Owing to the considerable biochemical diversity and structural complexity among different plant species and tissue types, numerous DNA isolation protocols have been optimized to remove major contaminants such as polyphenolic compounds, polysaccharides, and RNA and to ensure the efficient extraction of high-quality genomic DNA. The present investigation was conducted to compare and identify the efficient and suitable method that addresses the major challenges in extracting high-quality DNA from orchid tissues, which contain interfering mucilaginous compounds that can inhibit downstream applications. Here, three different methods, namely Method 1, Method 2, and Method 3, proposed by Cota-Sánchez *et al.* (2006), Kamba and Deb (2018) and Quintanilla-Quintero *et al.* (2011) are described respectively. These methods were systematically compared for the purity and yield of genomic DNA from leaves and roots of two orchid species, *Acampe praemorsa* and *Rynchostylis retusa*. This comparative analysis revealed Method 3 proposed by Quintanilla-Quintero *et al.,* (2011) as the most effective approach for extracting high-quality genomic DNA from orchids. Moreover, root tissues provided higher DNA quality and quantity than other plant tissues.

Keywords **:** Orchids, DNA extraction, *Acampe praemorsa*, *Rhynchostylis retusa,* CTAB, methodology, vandaceous.

Introduction

The Orchidaceae family represents the most diverse group of angiosperms, comprising an estimated 600 to 800 genera and 25,000 to 35,000 species worldwide. Among these, approximately 158 genera and 1,331 species are found in India and are known for their ornamental value and economic significance (Chen, 2009). *Acampe praemorsa* and *Rhynchostylis retusa* stand out as notable species, not only for their ornamental significance but also as key subjects in molecular biology studies. A. *praemorsa* has drawn considerable attention for its bioactive compounds, which show promising anticancer, antibacterial, antifungal, antioxidant, and anti-inflammatory properties (Vibha *et al.,* 2019). Meanwhile, R. *retusa* has been investigated for its genetic stability in micropropagation, utilizing RAPD markers (Oliya *et al.,* 2021).

These orchids provide a unique opportunity to explore genetic diversity and evolutionary relationships within the family, facilitated by advancements in DNA extraction and analysis techniques. *Acampe praemorsa*, commonly known as clipped Acampe or brittle orchid, is a species of monopodial orchid (Fig. 1A). It is native to tropical and subtropical Asia, specifically found in India, Sri Lanka, Thailand, and Burma. It is a robust epiphytic shrub, typically growing on trees. The stem is stout, measuring 20-50 cm in height, with vermiform roots emerging from basal nodes. Similarly, *Rhynchostylis retusa* (L.) Blume, another epiphytic member, is widely recognized for its striking pendant inflorescences adorned with pink-spotted white flowers (Fig. 1B). This species exhibits unique morphological

traits, such as specific leaf and root anatomy, which aid in its adaptation (Rajan *et al.,* 2024). The population of these species is declining due to habitat destruction and overexploitation, necessitating conservation efforts.

Table 1 : Comparative taxonomic features of *Acampe praemorsa* and *Rhynchostylis retusa*

Kingdom	Plantae	Plantae
Clade	Tracheophytes, Angiosperms	Tracheophytes, Angiosperms
Order	Asparagales	Asparagales
Family	Orchidaceae	Orchidaceae
Subfamily	Epidendroideae	Epidendroideae
Genus	Acampe	Rhynchostylis
Species	A. praemorsa	R. retusa
Subtribe	N/A	Aeridinae
Binomial name	*Acampe praemorsa* (Roxb.) Blatt. & McCann (1932)	*Rhynchostylis retusa* (L.) Blume

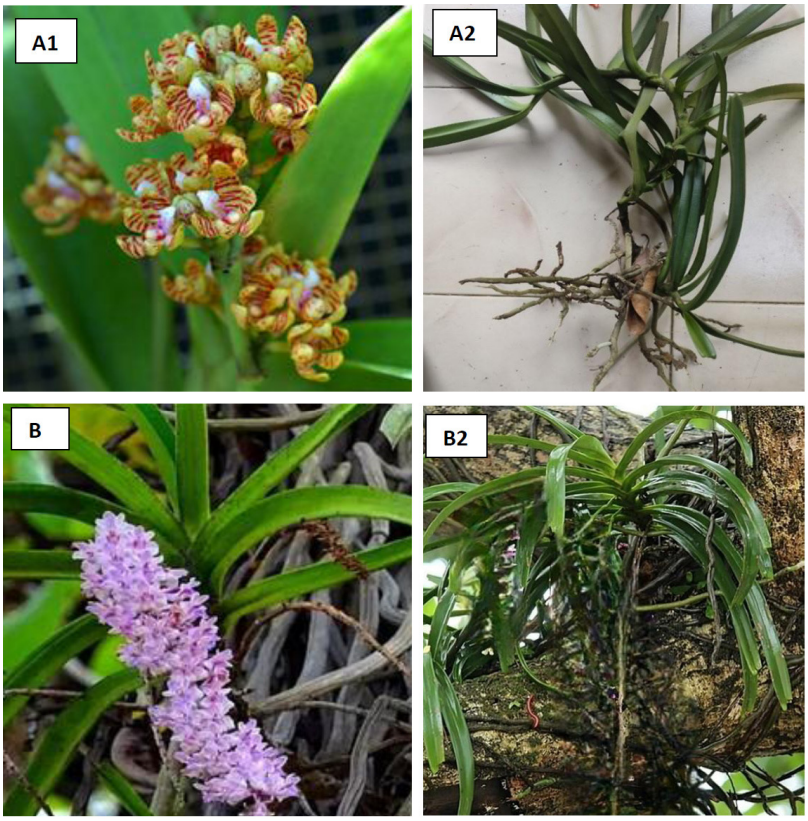

Fig. 1: Distinctive morphological features of *Acampe praemorsa* (A1, A2) and *Rhynchostylis retusa* (B1, B2)

Molecular studies play a crucial role in conservation by assessing genetic diversity, identifying endangered species, and guiding strategies for in situ and ex situ preservation of orchid species. Plant DNA extraction, and particularly the standardization of DNA extraction methods is fundamental to ensuring the reliability and reproducibility of downstream molecular biology applications in plant research, including phylogenetic analysis, genetic diversity studies, and DNA barcoding. Interfering compounds can suppress PCR and restriction enzyme activity (Fang *et al.,* 1992), which is necessary for subsequent analysis. However, the diverse nature of plant tissues, the presence of various contaminants, and the challenges associated with different sample types necessitate careful consideration and optimization of extraction methodologies. The optimal DNA isolation procedure is highly dependent on the specific plant species and tissue type. While numerous methods and kits are described, the latter can be costly and are typically organism-specific (Hoarau *et al.,* 2007; Ahmed *et al.,* 2009; Margam *et al.,* 2010).

While working with these vandaceous orchids, which represent a valuable resource for extending the temporal and taxonomic scope of molecular studies, researchers face the additional challenges of DNA degradation, contamination with microbial DNA, and the presence of chemical fixatives.

In an attempt to carry out the metagenomic analysis of endophytic fungi associated with vandaceous orchids, we tried to isolate the total DNA from *Acampe praemorsa* and *Rhynchostylis retusa* using normal laboratory methods. We could not get good-quality DNA with the normal methods, and we understood that plant DNA extraction in orchids is filled with unique challenges due to high levels of interfering polyphenols, polysaccharides, and other secondary metabolites.

Various DNA extraction methods have been developed and refined to address these issues. The present study was designed for comparing and evaluating three extraction methods on the described species, which was collected from different parts of Kerala, and to determine the best method for DNA extraction and amplification. The cetyltrimethylammonium bromide (CTAB) method is a widely used approach for plant DNA extraction, often modified to enhance its efficacy for specific plant groups or sample types. We have made significant modifications (Table 1), making this method a practical alternative to other laborious and expensive protocols.

Materials and Methods

Plant material and collection

The vandaceous orchids (Fig. 1) used for extraction have been collected from different parts of the Kerala, India. For each species, both leaf and root tissues were sampled from healthy, mature individuals. A total of 36 samples were prepared, representing the factorial design: 2 species × 2 tissue types × 3 extraction methods × 3 biological replicates. The samples were collected from natural habitats where no formal collection permit was required, and the work was conducted strictly for non-commercial academic research.

The plant tissues were surface sterilized before extraction following Deb and Imchen (2010). Samples were immersed in 70% ethanol for 60 s, followed by 4% sodium hypochlorite for 10 min, and rinsed three times with sterile distilled water. The cleaned tissues were blotted dry, cut into small pieces using sterile scissors, and stored at 4 °C until DNA extraction.

Chemical Reagents

All chemicals were of molecular-biology grade (HiMedia, Thermo Fisher Scientific). The general extraction reagents included:

CTAB extraction buffer (stocks of 1M Tris-HCl (pH 8.0), 5M NaCl, 0.5M EDTA (pH 8.0), 2% CTAB)

Dithiothreitol (DTT): A strong reducing agent. Its primary role is to inactivate degradative enzymes and prevent oxidative damage.

PVP: A water-soluble polymer. Its primary role is to remove secondary metabolites, especially polyphenols.

β-mercaptoethanol: reduces oxidative damage to DNA by neutralizing reactive oxygen species.

Phenol: Chloroform (1:1) and Chloroform: Isoamyl alcohol (24:1): used sequentially for phase separation, with the former denaturing proteins and the latter removing residual lipids and polysaccharides.

Chilled isopropanol: precipitation of DNA

Tris-EDTA (TE buffer) (10 mM Tris, pH 8.0, 0.1 mM EDTA): storage of DNA

Ethanol 70% and 90%: washing precipitated DNA to remove salts and residual organic contaminants.

7.5 M ammonium acetate: To enhance DNA precipitation

All buffers were freshly prepared, and organic solvents were equilibrated before use.

Extraction methodology

A comparative evaluation of three genomic DNA extraction methods was conducted to identify the most efficient and reproducible protocol for vandaceous orchids. To evaluate extraction efficiency, three established CTAB-based methods Cota-Sánchez *et al*. (2006), Kamba and Deb (2018), and Quintanilla-Quintero *et al*. (2011) were tested with minor modifications (Table 2) to optimize yield and purity for orchid tissues. Each extraction was performed in triplicate for every species and tissue combination.

Table 2 : Sequential representation of the three extraction methods

	Method 1 Cota-Sánchez *et al.* (2006)	**Method 2 Kamba and Deb (2018)**	**Method 3 Quintanilla-Quintero *et al.* (2011)**
Sample preparation	1.0 g plant tissue, grounded with liquid nitrogen and sterilized sand	200 mg orchid leaf tissue, ground in pre-chilled mortar, no use of liquid nitrogen	1g plant tissue grounded with liquid nitrogen
CTAB Buffer composition	1.0 M Tris-HCl, 5 M NaCl, 0.25 M EDTA (pH 8.0)	1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, and 2% CTAB	0.1M Tris-HCl (pH 8.0), 1.4M NaCl, 0.02M EDTA (pH 8.0), 2% CTAB, 0.7% v/v DTT, 2% soluble PVP
Extraction solvents	Chloroform:Isoamyl (24:1) × 2	Chloroform:Isoamyl (24:1) × 2	Phenol:Chloroform (1:1) → Chloroform:Isoamyl (24:1)
Precipitation	0.33 vol isopropanol (-20 °C, overnight)	Isopropanol (-20 °C, ≥1 h)	0.6 vol isopropanol + 100 µL 7.5 M NH_4OAc (-20 °C, overnight)

Method 1: Cota-Sánchez *et al.,* (2006)

Approximately 1 g of young leaf or root tissue was ground to a fine powder with liquid nitrogen using a sterile mortar and pestle with a small quantity of sterilized sand to aid mechanical disruption. The powdered tissue was transferred into a sterile 2 mL microcentrifuge tube containing 750 µL of pre-heated 2X CTAB extraction buffer (1 M Tris-HCl, 5 M NaCl, 0.25 M EDTA, pH 8.0, and 2% CTAB). Immediately before incubation, 3 µL of β-mercaptoethanol was added to each tube. Samples were incubated at 65 °C for 1-2 h, with occasional gentle inversion.

After incubation, 700 µL of chloroform:isoamyl alcohol (24:1) was added to each tube, mixed thoroughly by inversion, and centrifuged at 10,000 rpm for 15 min at 4 °C. The upper aqueous phase was carefully transferred to a new tube and re-extracted once more with an equal volume of chloroform: isoamyl alcohol to remove residual contaminants. DNA was precipitated by adding 0.33 volumes of chilled isopropanol, followed by gentle mixing and incubation at -20 °C overnight. The DNA was pelleted by centrifugation at 14,000 rpm for 5 min, washed with 70% ethanol, air-dried, and finally resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Method 2: Kamba and Deb (2018)

This modified CTAB protocol was optimized to eliminate the use of phenol and liquid nitrogen, making it safer and more cost-effective while maintaining good DNA quality. Approximately 200 mg of tissue was homogenized in a pre-chilled mortar with 2 mL of CTAB buffer (1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, 2% CTAB, and 2% PVP w/v). β-mercaptoethanol (0.2%) was added immediately before grinding to prevent oxidation of polyphenols. The homogenate was transferred to 2 mL tubes and incubated at 60 °C for 35 min, with occasional gentle mixing.

After incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added, and the samples were centrifuged at 10,000 rpm for 15 min at 4 °C to separate the aqueous and organic phases. The upper aqueous layer was carefully collected into a new tube, and DNA was precipitated by adding an equal volume of chilled isopropanol. The mixture was inverted gently and incubated at -20 °C overnight. DNA was pelleted by centrifugation at 14,000 rpm for 10 min, washed with 70% ethanol, air-dried, and finally dissolved in TE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Method 3: Quintanilla-Quintero *et al.,* (2011)

This protocol combines phenol–chloroform and chloroform–isoamyl extractions with CTAB buffer containing strong reducing and complexing agents to remove phenolics and polysaccharides. Approximately 1 g of tissue was ground in liquid nitrogen to a fine powder and transferred into 2 mL microcentrifuge tubes containing 1.5 mL of preheated (65 °C) CTAB buffer (0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, 0.7% DTT, and 2% PVP). 50 µL of β-mercaptoethanol was added to each tube before incubation.

Samples were incubated at 65 °C for 30 min with occasional gentle mixing to lyse cells. The lysate was then extracted with an equal volume of phenol:chloroform (1:1) and centrifuged at 16,000 rpm for 15 min at 4 °C. The aqueous phase was carefully transferred to a new tube and extracted again with chloroform:isoamyl alcohol (24:1) to remove any remaining phenolic compounds.

DNA was precipitated by adding 0.6 volume of chilled isopropanol and 100 µL of 7.5 M ammonium acetate, mixed gently, and incubated at -20 °C overnight. The resulting DNA pellet was collected by

centrifugation at 14,000 rpm for 5 min, washed with 70% ethanol, air-dried, and resuspended in TE buffer.

DNA quantification and quality assessment

DNA concentration and purity were determined using a NanoDrop UV spectrophotometer by recording absorbance at 260/280 nm and 260/230 nm. Integrity of DNA was verified by loading on 0.8% agarose gels in 1X TAE buffer, stained with ethidium bromide and visualized under UV light.

PCR validation using ISSR primer (UBC 807)

To evaluate the suitability of extracted DNA for downstream applications, PCR amplification was performed using the ISSR primer UBC 807. Each 25 µL PCR reaction contained: 1 X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primer, 0.5 U *Taq* DNA polymerase (Thermo Fisher), and 25-50 ng template DNA.

The amplification program consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 90 s, with a final extension at 72 °C for 7 min. The amplification products were separated on 1.5% agarose gels, stained with ethidium bromide, and observed under gel documentation system.

Statistical analysis

All data analyses were conducted using R version 4.4.3. ANOVA was performed to assess the effects of species (2 levels), tissue type (2 levels), and extraction method (3 levels) on DNA yield and purity (A260/280 and A260/230). Post-hoc comparisons were made using Tukey's HSD test at $\alpha = 0.05$. Results are reported as mean ± standard deviation (SD).

Results

Tissue homogenization

The efficiency of tissue pulverization significantly influenced the quality of DNA extraction from orchidaceous samples. Among the three protocols evaluated, Method 1 (Cota-Sánchez *et al*., 2006) and Method 3 (Quintanilla-Quintero *et al*., 2011) incorporated liquid nitrogen-assisted grinding, which facilitated complete pulverization of plant tissues. This approach resulted in a fine powder that enabled efficient cell wall disruption and enhanced extraction buffer penetration. In contrast, Method 2 (Kamba and Deb, 2018) employed conventional mortar and pestle grinding at ambient temperature.

The liquid nitrogen-based pulverization, although requiring additional time and resources, proved essential for orchid tissues due to their unique anatomical characteristics. Methods 1 and 3 produced finer, more uniform powder compared to Method 2, which resulted in partially intact tissue fragments and inconsistent particle sizes.

Comparative analysis of DNA yield

The three extraction protocols demonstrated significant variations in DNA recovery efficiency across both orchid species and tissue types (Table 3). Analysis of variance revealed highly significant differences among methods ($F = 45.32$, $p < 0.001$), tissue types ($F = 28.67$, $p < 0.001$), and their interactions ($F = 12.45$, $p < 0.01$). Method 3 (Quintanilla-Quintero *et al*., 2011) consistently outperformed the other protocols, yielding DNA concentrations ranging from 599.7 to 794.43 ng/µL across all experimental combinations, with a mean concentration of 690.39 ± 138.29 ng/µL, representing a 47.1% increase over Method 1 and a 105.5% increase over Method 2. Method 1 (Cota-Sánchez *et al*., 2006) produced intermediate yields ranging from 412.13 to 533.4 ng/µL (469.33 ± 68.88 ng/µL), while Method 2 (Kamba and Deb, 2018) yielded the lowest concentrations, ranging from 280.37 to 401.6 ng/µL (335.93 ± 56.42 ng/µL).

For *Acampe praemorsa*, Method 3 extracted 624.87 ± 116.97 ng/µL from leaf tissue and 742.57 ± 148.76 ng/µL from root tissue, significantly exceeding Method 1 (423.77 ± 53.46 ng/µL and 508.27 ± 68.02 ng/µL) and Method 2 (280.37 ± 37.66 ng/µL and 342.63 ± 45.17 ng/µL). Similarly, *Rhynchostylis retusa* yielded 599.7 ± 110.68 ng/µL from leaves and 794.43 ± 140.64 ng/µL from roots with Method 3, surpassing Method 1 (412.13 ± 59.83 ng/µL and 533.4 ± 61.94 ng/µL) and Method 2 (309.53 ± 40.46 ng/µL and 401.6 ± 33.98 ng/µL). Notably, *Rhynchostylis retusa* root tissue processed through Method 3 achieved the maximum DNA concentration (794.43 ± 140.64 ng/µL), while *Acampe praemorsa* leaf tissue extracted using Method 2 yielded the minimum (280.37 ± 37.66 ng/µL).

Root tissues consistently yielded higher DNA concentrations than leaf tissues across all methods and species, producing 19.9%, 22.2%, and 18.8% more DNA than leaves in Methods 1, 2, and 3, respectively (paired t-test, $p < 0.01$). For *Acampe praemorsa*, roots exceeded leaf yields by 20.0%, 22.2%, and 18.8% in Methods 1, 2, and 3, while *Rhynchostylis retusa* showed increases of 29.4%, 29.8%, and 32.5%.

Table 3 : Comparison of DNA concentration and purity obtained from leaf and root tissues of *Acampe praemorsa* and *Rhynchostylis retusa* using three different extraction methods.

Method	Species	Tissue	DNA Conc. (ng/µL)	A260/A280	A260/A230
Method 1	*Acampe praemorsa*	Leaf	423.77 ± 53.46^{abc}	1.75 ± 0.04^{abc}	1.71 ± 0.06^{ab}
		Root	508.27 ± 68.02^{bc}	1.74 ± 0.06^{bcd}	1.77 ± 0.07^{ab}
	Rhynchostylis retusa	Leaf	412.13 ± 59.83^{bc}	1.78 ± 0.04^{ab}	1.77 ± 0.08^{ab}
		Root	533.4 ± 61.94^{bc}	1.77 ± 0.05^{abc}	1.81 ± 0.08^{ab}
Method 2	*Acampe praemorsa*	Leaf	280.37 ± 37.66^{c}	1.65 ± 0.03^{c}	1.54 ± 0.06^{b}
		Root	342.63 ± 45.17^{c}	1.64 ± 0.04^{d}	1.56 ± 0.06^{b}
	Rhynchostylis retusa	Leaf	309.53 ± 40.46^{c}	1.66 ± 0.04^{bc}	1.58 ± 0.05^{b}
		Root	401.6 ± 33.98^{c}	1.68 ± 0.04^{cd}	1.64 ± 0.06^{b}
Method 3	*Acampe praemorsa*	Leaf	624.87 ± 116.97^{a}	1.85 ± 0.05^{a}	1.9 ± 0.16^{a}
		Root	742.57 ± 148.76^{ab}	1.87 ± 0.05^{ab}	1.95 ± 0.16^{a}
	Rhynchostylis retusa	Leaf	599.7 ± 110.68^{ab}	1.86 ± 0.06^{a}	1.95 ± 0.17^{a}
		Root	794.43 ± 140.64^{a}	1.89 ± 0.06^{a}	2.02 ± 0.17^{a}

Values represent mean ± standard deviation (n = 3). Means followed by different superscript letters within a column differ significantly according to Tukey's HSD test ($p < 0.05$). Method 3 yielded significantly higher DNA concentrations and purity ratios (A260/A280 and A260/A230) compared with Methods 1 and 2.

DNA purity assessment

The spectrophotometric purity assessment revealed significant differences among extraction methods in both protein and organic contaminant levels. The A260/A280 ratio, indicative of protein contamination with optimal values between 1.8 and 2.0, showed Method 3 producing ratios of 1.85-1.89 (1.87 ± 0.02), significantly exceeding Method 1 (1.71-1.81; 1.76 ± 0.02) and Method 2 (1.54-1.68; 1.66 ± 0.02) according to Tukey's HSD test ($p < 0.05$). The A260/A230 ratio, reflecting contamination by polysaccharides, polyphenols, and other organic compounds, demonstrated similar superiority for Method 3 (1.90-2.02; 1.96 ± 0.05), with root samples from *Rhynchostylis retusa* achieving the optimal value of 2.02 ± 0.17. These values significantly exceeded Method 1 (1.71-1.81; 1.76 ± 0.05) and Method 2 (1.54-1.64; 1.59 ± 0.04), as confirmed by ANOVA analysis.

DNA integrity assessment

The visual assessment of DNA integrity through 0.8% agarose gel electrophoresis revealed distinct qualitative differences among the three extraction methods (Figures 2a and 2b, Table 4). The DNA samples extracted from *Acampe praemorsa* and *Rhynchostylis retusa* exhibited clear, distinct bands indicative of high molecular weight genomic DNA, though band intensity and integrity varied considerably among methods.

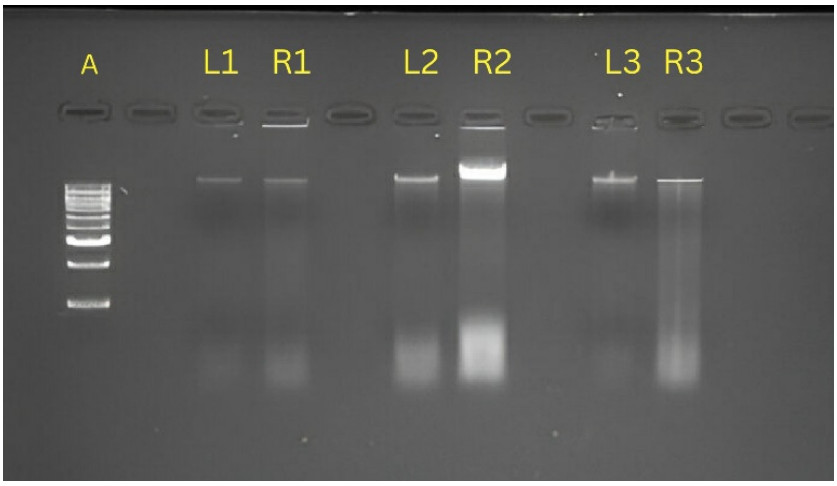

Fig. 2a : Gel electrophoresis comparing genomic DNA extracted from leaf tissues (L1, L2, L3) and root tissues (R1, R2, R3) of *Rhynchostylis retusa* with a 1kb ladder (A). L1, R1 represents Method 1; L3, R3 represents Method 2; L2, R2 represents Method 3

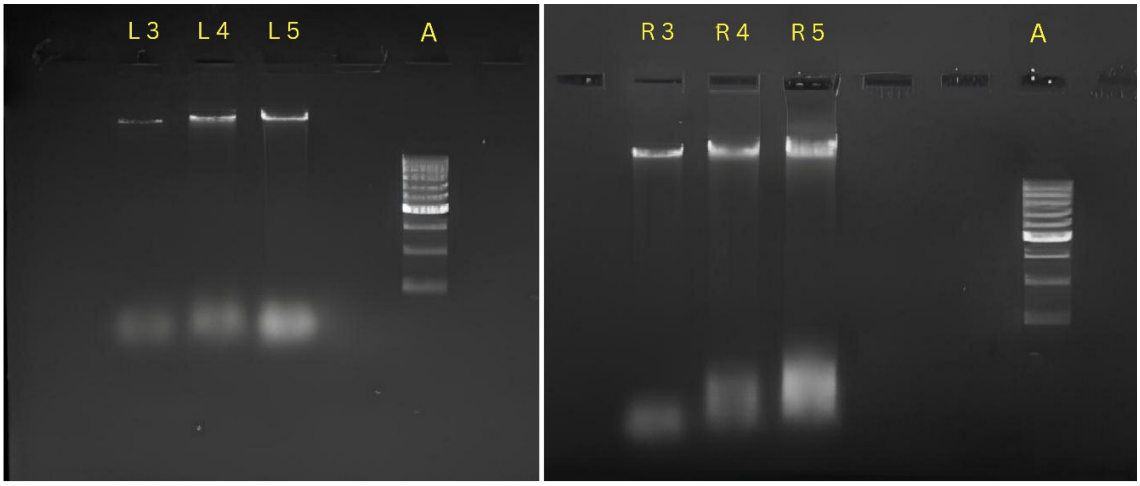

Fig. 2b : Gel electrophoresis comparing genomic DNA extracted from leaf tissues (L4, L5, L6) and root tissues (R4, R5, R6) of *Acampe praemorsa* with a 1kb ladder (A). L4, R4 represents Method 1; L5, R5: represents Method 2; L6, R6: represents Method 3

For *Rhynchostylis retusa* (Figure 2a), Method 3 (lanes L2 and R2) produced the most superior results, with very high band intensity in root samples and high intensity in leaf samples. The bands were sharp and intact with no visible smearing or degradation, indicating genomic DNA of excellent quality with minimal nuclease activity during extraction. Method 1 (lanes L1 and R1) showed high band intensity in root samples and moderate intensity in leaf samples, with sharp bands and minimal smearing, demonstrating good but slightly inferior DNA quality compared to Method 3. Method 2 (lanes L3 and R3) produced moderate band intensity in root samples and low intensity in leaf samples, with diffuse bands and some visible degradation, particularly in leaf tissue.

Similar patterns were observed for *Acampe praemorsa* (Figure 2b). Method 3 (lanes L6 and R6) again demonstrated superior performance with very high band intensity in root samples and high intensity in leaf samples, exhibiting sharp, intact bands without trailing or degradation. Method 1 (lanes L4 and R4) showed high band intensity in root samples and moderate intensity in leaf samples with clear bands and minor smearing. Method 2 (lanes L5 and R5) produced the weakest results, with moderate band intensity in root samples and low intensity in leaf samples, accompanied by diffuse bands and moderate smearing indicative of partial DNA degradation.

Table 4 : Qualitative Assessment of DNA Integrity on 0.8% Agarose Gel

Method	Sample Type	Band Intensity	Smearing	Observations
Method 1 Cota-Sánchez *et al.* (2006)	Leaf	Moderate	Low	Clear bands, minor smearing
	Root	High	Minimal	Sharp bands, no visible degradation
Method 2 Kamba and Deb (2018)	Leaf	Low	Moderate	Diffuse bands, some degradation
	Root	Moderate	Low	Clearer bands, minimal degradation
Method 3 Quintanilla-Quintero *et al.* (2011)	Leaf	High	Minimal	Sharp, intact bands
	Root	Very High	None	Very sharp, intact bands, no trailing

The incorporation of an ammonium acetate and 2-propanol purification step, particularly emphasized in Method 3, significantly enhanced both pellet visibility and DNA recovery. This additional purification step resulted in an approximate 15-20% increase in yield compared to protocols lacking this step, while simultaneously improving purity.

Validation through PCR amplification

To assess the suitability of extracted DNA for downstream molecular applications, PCR amplification was performed using the inter-simple sequence repeat (ISSR) primer UBC 807. All DNA samples, regardless of extraction method, yielded successful amplification products, confirming the absence of absolute PCR inhibition. However, amplification efficiency varied

considerably among methods, correlating strongly with the observed purity indices.

DNA extracted using Method 3 produced the most intense and reproducible banding patterns, with consistent amplification across samples. Method 1-derived DNA showed moderate amplification efficiency with slightly reduced band intensity, while Method 2-derived DNA, despite successful amplification, exhibited the weakest bands and occasional amplification failures in replicate reactions. These observations align with the purity measurements, reinforcing the conclusion that Method 3 produces DNA of superior quality for downstream analysis.

Discussion

The present study demonstrates that Method 3 (Quintanilla-Quintero *et al*., 2011) consistently outperformed alternative protocols in extracting high-quality genomic DNA from vandaceous orchids, achieving mean concentrations of 690.39 ng/µL with superior purity indices (A260/A280: 1.87; A260/A230: 1.96). This performance advantage can be attributed primarily to the synergistic effects of cryogenic tissue homogenization and enhanced purification strategies that effectively mitigate the biochemical challenges inherent to orchidaceous tissues.

The superiority of liquid nitrogen-assisted grinding observed in Methods 1 and 3 aligns with established principles of plant DNA extraction, particularly for tissues rich in secondary metabolites and mucilaginous compounds (Khanuja *et al*., 1999; Varma *et al*., 2007). Cryogenic pulverization achieves three critical objectives: it rapidly inactivates endogenous nucleases that would otherwise degrade DNA during cell disruption (Porebski *et al*., 1997), prevents enzymatic oxidation of polyphenolic compounds that can irreversibly bind to and coprecipitate with nucleic acids (Pandey *et al*., 1996), and renders mucilaginous tissues brittle, facilitating more complete cellular disruption (Loomis, 1974). The anatomical characteristics of vandaceous orchids-including abundant mucilage cells, specialized velamen tissue in roots, and high concentrations of phenolic compounds make these considerations particularly critical (Benzing *et al*., 1982).

The ambient-temperature grinding employed in Method 2 resulted in significantly lower yields (335.93 ng/µL) and compromised purity ratios, findings consistent with previous reports demonstrating that phenolic oxidation during tissue disruption forms quinones that covalently crosslink with DNA, resulting in reduced yields and brownish discoloration of extracts (John, 1992; Maniatis, 1982).

The exceptional performance of Method 3 can be mechanistically attributed to its modified purification strategy incorporating ammonium acetate precipitation followed by 2-propanol. Ammonium acetate selectively retains proteins and polysaccharides in solution while allowing nucleic acids to precipitate with alcohol, thereby achieving superior removal of contaminants compared to ethanol precipitation (Maniatis, 1982). This is particularly relevant for orchid tissues, which contain high concentrations of mucopolysaccharides that precipitate along with the DNA (Novak *et al*., 2014; Salazar *et al*., 2003).

The elevated A260/A230 ratios achieved by Method 3 (1.96) compared to Methods 1 (1.76) and 2 (1.59) reflect effective removal of carbohydrates and phenolic compounds that absorb strongly at 230 nm (Barbas *et al*., 2007; Wilfinger *et al*., 1997). Previous studies on recalcitrant plant species have similarly reported that ammonium acetate precipitation significantly improves A260/A230 ratios by eliminating polysaccharide contamination (Healey *et al*., 2014). The improved A260/A280 ratios in Method 3 indicate reduced protein contamination, considered optimal for pure DNA preparations (Gallagher and Desjardins, 2006; Glasel, 1995).

The present findings have particular relevance for molecular systematic studies seeking to resolve phylogenetic relationships within the species-rich Vandeae tribe, which comprises over 2,000 species distributed across 130+ genera (Givnish *et al*., 2015). Previous phylogenetic analyses have been hampered by DNA quality issues, particularly for plastid genome sequencing and phylogenomic approaches requiring large amounts of high-quality, high molecular weight DNA (Givnish *et al*., 2015; Li *et al*., 2019). Method 3's consistent delivery of high-quality DNA suitable for next-generation sequencing platforms could facilitate genome-scale phylogenetic analyses that are necessary to resolve remaining systematic uncertainties in this diverse clade.

Additionally, the recommendation for preferential root tissue sampling aligns well with conservation ethics, as root collection from cultivated specimens causes minimal harm compared to destructive leaf sampling from wild populations.

Conclusion

This comprehensive evaluation establishes Method 3 (Quintanilla-Quintero *et al*., 2011) as the optimal protocol for extracting high-quality genomic DNA from vandaceous orchids, achieving superior

performance across yield, purity, integrity, and downstream applications. The protocol's success stems from synergistic incorporation of cryogenic tissue homogenization which prevents phenolic oxidation and nuclease activity with ammonium acetate-based purification that effectively removes polysaccharide and protein contaminants characteristic of orchidaceous tissues.

The consistent superiority of root versus leaf tissues across all protocols provides guidance for researchers, suggesting that root tissue should be preferentially targeted for DNA extraction in vandaceous orchids when high-quality DNA is required. This recommendation aligns with conservation considerations, as root sampling from cultivated specimens minimizes harm compared to destructive foliar sampling from wild populations.

These findings provide an evidence-based foundation for standardizing DNA extraction protocols in orchid molecular research, potentially enhancing reproducibility, data quality, and success rates for demanding applications including next-generation sequencing, population genetics, and conservation genomics. As molecular approaches become increasingly central to orchid taxonomy, systematics, and conservation, the availability of reliable, validated protocols becomes ever more critical for advancing our understanding and protection of this diverse and threatened family.

Supplementary Information

Supplementary Table S1. ANOVA results for DNA yield and purity indices across species, tissue types, and extraction methods.

Supplementary Fig. S1. PCR amplification profiles of DNA extracted using three methods showing ISSR banding patterns.

Supplementary material referenced in the text is provided as separate file along with this manuscript.

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Author contribution

S Bharathmithran: Investigation and Methadology, Original draft preparation, Dr R Preetha: Conceptualization, Supervision, Review and editing.

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Declarations

Ethical approval: “Not applicable”.

Informed consent: “Not applicable”.

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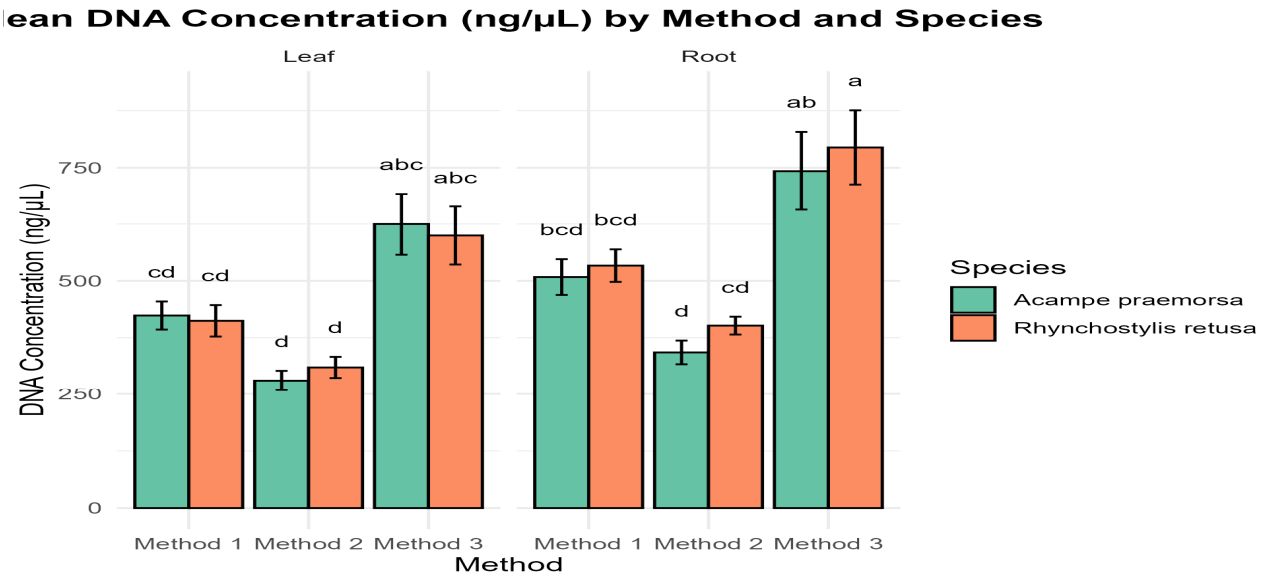
Supplementary Table S1. ANOVA results for DNA yield and purity indices across species, tissue types, and extraction methods.

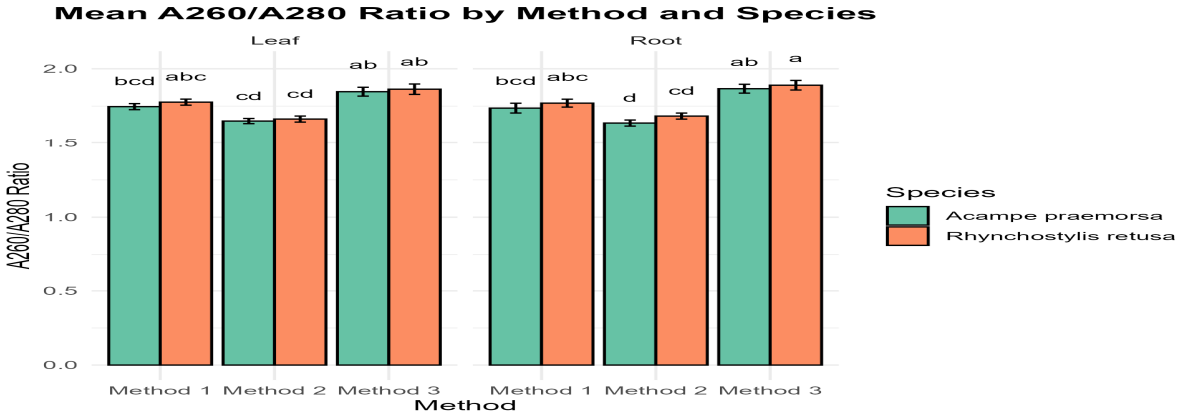
DNA Concentration (ng/µL)

Species	Method	Tissue	Mean ± SD (group)	F-stat	CV (%)	SE	SM
Acampe praemorsa	Method 1	Leaf	423.77 ± 53.46cd	0.555	17.3	30.86348	17.81904
Acampe praemorsa	Method 1	Root	508.27 ± 68.02bcd	0.555	17.3	39.27265	22.67408
Acampe praemorsa	Method 2	Leaf	280.37 ± 37.66d	0.555	17.3	21.74491	12.55443
Acampe praemorsa	Method 2	Root	342.63 ± 45.17d	0.555	17.3	26.07811	15.05620
Acampe praemorsa	Method 3	Leaf	624.87 ± 116.97abc	0.555	17.3	67.53343	38.99045
Acampe praemorsa	Method 3	Root	742.57 ± 148.76ab	0.555	17.3	85.88477	49.58560
Rhynchostylis retusa	Method 1	Leaf	412.13 ± 59.83cd	0.555	17.3	34.54380	19.94387
Rhynchostylis retusa	Method 1	Root	533.4 ± 61.94bcd	0.555	17.3	35.76143	20.64687
Rhynchostylis retusa	Method 2	Leaf	309.53 ± 40.46d	0.555	17.3	23.35811	13.48581
Rhynchostylis retusa	Method 2	Root	401.6 ± 33.98cd	0.555	17.3	19.61666	11.32568
Rhynchostylis retusa	Method 3	Leaf	599.7 ± 110.68abc	0.555	17.3	63.89901	36.89211
Rhynchostylis retusa	Method 3	Root	794.43 ± 140.64a	0.555	17.3	81.19789	46.87963



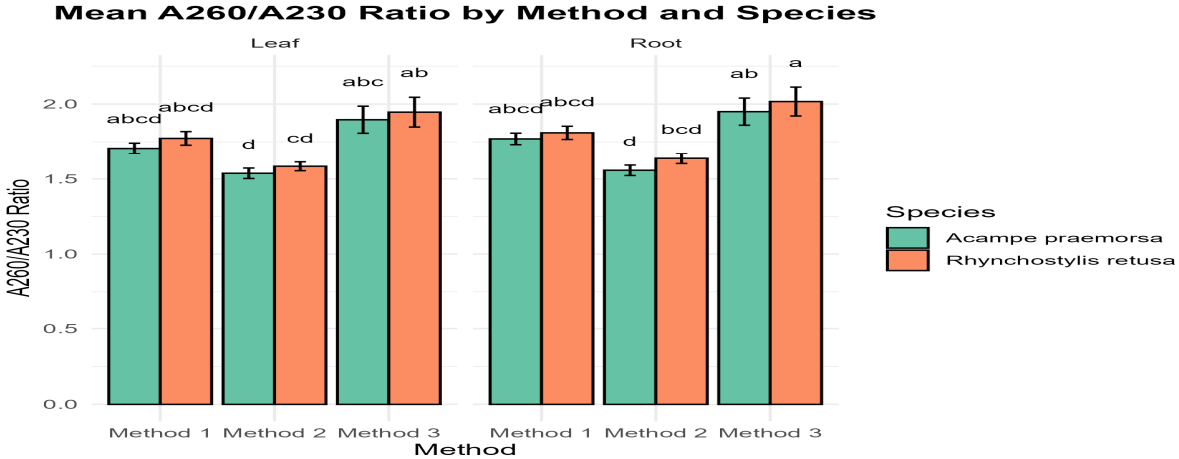
A260/A280 Ratio

Species	Method	Tissue	Mean ± SD (group)	F-stat	CV (%)	SE	SM
Acampe praemorsa	Method 1	Leaf	1.75 ± 0.04bcd	3.28	2.561	0.02027588	0.01170628
Acampe praemorsa	Method 1	Root	1.74 ± 0.06bcd	3.28	2.561	0.03282953	0.01895414
Acampe praemorsa	Method 2	Leaf	1.65 ± 0.03cd	3.28	2.561	0.01732051	0.01000000
Acampe praemorsa	Method 2	Root	1.64 ± 0.04d	3.28	2.561	0.02027588	0.01170628
Acampe praemorsa	Method 3	Leaf	1.85 ± 0.05ab	3.28	2.561	0.02962731	0.01710534
Acampe praemorsa	Method 3	Root	1.87 ± 0.05ab	3.28	2.561	0.02962731	0.01710534
Rhynchostylis retusa	Method 1	Leaf	1.78 ± 0.04abc	3.28	2.561	0.02027588	0.01170628
Rhynchostylis retusa	Method 1	Root	1.77 ± 0.05abc	3.28	2.561	0.02645751	0.01527525
Rhynchostylis retusa	Method 2	Leaf	1.66 ± 0.04cd	3.28	2.561	0.02027588	0.01170628
Rhynchostylis retusa	Method 2	Root	1.68 ± 0.04cd	3.28	2.561	0.02027588	0.01170628
Rhynchostylis retusa	Method 3	Leaf	1.86 ± 0.06ab	3.28	2.561	0.03480102	0.02009238
Rhynchostylis retusa	Method 3	Root	1.89 ± 0.06a	3.28	2.561	0.03214550	0.01855921



A260/A230 Ratio

Species	Method	Tissue	Mean ± SD (group)	F-stat	CV (%)	SE	SM
Acampe praemorsa	Method 1	Leaf	1.71 ± 0.06abcd	2.673	6.064	0.03480102	0.02009238
Acampe praemorsa	Method 1	Root	1.77 ± 0.07abcd	2.673	6.064	0.03785939	0.02185813
Acampe praemorsa	Method 2	Leaf	1.54 ± 0.06d	2.673	6.064	0.03480102	0.02009238
Acampe praemorsa	Method 2	Root	1.56 ± 0.06d	2.673	6.064	0.03480102	0.02009238
Acampe praemorsa	Method 3	Leaf	1.9 ± 0.16abc	2.673	6.064	0.08950481	0.05167563
Acampe praemorsa	Method 3	Root	1.95 ± 0.16ab	2.673	6.064	0.08962886	0.05174725
Rhynchostylis retusa	Method 1	Leaf	1.77 ± 0.08abcd	2.673	6.064	0.04484541	0.02589151
Rhynchostylis retusa	Method 1	Root	1.81 ± 0.08abcd	2.673	6.064	0.04358899	0.02516611
Rhynchostylis retusa	Method 2	Leaf	1.58 ± 0.05cd	2.673	6.064	0.02962731	0.01710534
Rhynchostylis retusa	Method 2	Root	1.64 ± 0.06bcd	2.673	6.064	0.03480102	0.02009238
Rhynchostylis retusa	Method 3	Leaf	1.95 ± 0.17ab	2.673	6.064	0.09820613	0.05669934
Rhynchostylis retusa	Method 3	Root	2.02 ± 0.17a	2.673	6.064	0.09527737	0.05500842



Supplementary Fig. S1. PCR amplification profiles of DNA extracted using three methods showing ISSR banding patterns with primer UBC 807.

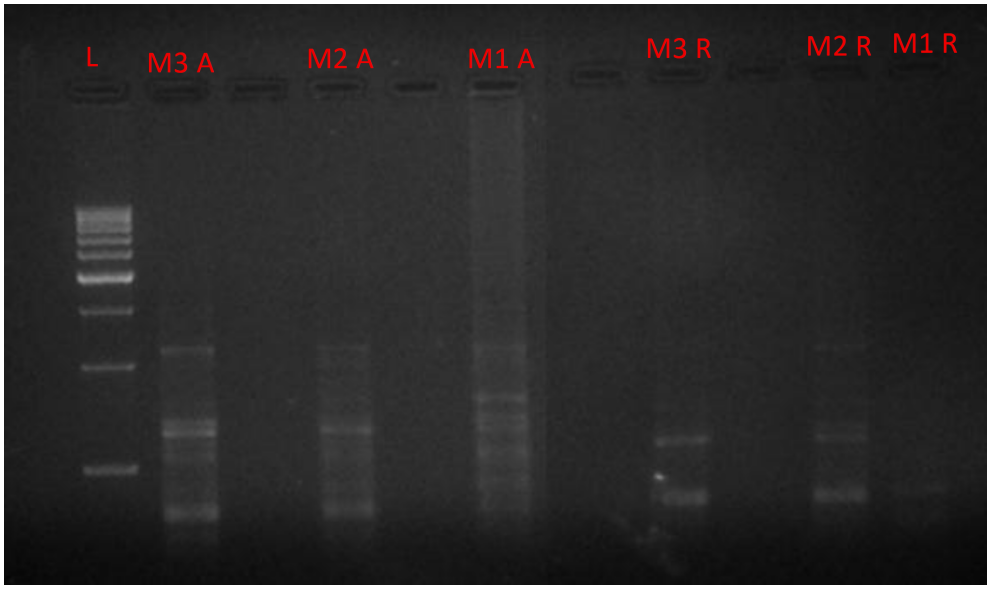


Fig S1. Gel electrophoresis comparing amplified PCR products of *Acampe praemorsa* (A) and *Rynchostylis retusa* (R) with a 1kb ladder. M1 represents Method 1; M2 represents Method 2; M3 represents Method 3