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From Shell to Sequence: Optimizing DNA Extraction and PCR for Pen Shell Identification

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Abstract: *Pinna nobilis*, an ecologically significant and critically endangered bivalve endemic to the Mediterranean Sea, has been classified as "Critically Endangered" by IUCN due to habitat degradation, climate change, and mass mortality events caused by the protozoan parasite *Haplosporidium pinnae*. Effective conservation efforts require robust molecular tools for species identification and genetic monitoring, necessitating the development of optimized DNA extraction and amplification protocols for a non-invasive sampling protocol. In this study, we evaluated multiple DNA extraction methods—Chelex-100, the sodium chloride (NaCl) method, a modified CTAB protocol, and a commercial kit, NucleoSpin Tissue Kit—using minute shell fragments from both ethanol-preserved and air-dried (dead) samples. We optimized key parameters, including incubation times, temperatures, and sample preparation, to determine the most effective protocol for obtaining high-quality DNA suitable for downstream applications. Additionally, we assessed different PCR strategies, including nested and semi-nested approaches targeting the *COI* gene marker, to enhance species identification. To further refine the methodology, we evaluated novel specific primers for nested PCR, improving sensitivity and specificity in detecting *P. nobilis* DNA from minute and degraded samples. Our results provide an optimized, cost-effective, and time-efficient workflow for non-invasive molecular identification of *P. nobilis*, with broad implications for conservation genetics, biodiversity monitoring, and species recovery programs.

Keywords: DNA extraction; *COI* barcoding; *Pinna nobilis*; conservation; PCR assays

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1. Introduction

1.1. Pinna nobilis: Ecological Role and Conservation Challenges

Pinna nobilis (the noble pen shell) is the largest bivalve mollusc endemic to the Mediterranean Sea, including the waters surrounding Greece. It is one of the largest bivalves in the world, reaching lengths of up to 120 cm. *Pinna nobilis* plays a vital ecological role, providing habitat for a wide range of marine species and contributing to the overall health of marine ecosystems [1]. Historically, its populations thrived in seagrass beds, particularly within *Posidonia oceanica* meadows, which provide an ideal substrate for the bivalves to anchor [2,3].

Since the 1970s, *P. nobilis* populations have rapidly declined due to anthropogenic activities, such as coastal construction and illegal harvesting, which have also resulted in the degradation of its habitat [4,5]. By the end of the 1990s, *P. nobilis* was classified as "vulnerable" on the International Union for Conservation of Nature (IUCN) Red List and included in the Annex of the EU Habitats Directive (92/43/CEE), constraining its conservation and harvesting. Despite these protective measures, *P. nobilis* populations have declined over the last decade, likely due to environmental stressors such as heatwaves, which may be linked to climate change [3]. This species has also suffered from mass mortality events (MMEs) in recent years, which have decimated *P. nobilis* populations across the Mediterranean since 2016 [6]. The primary driver of these MMEs has been the invasive protozoan parasite *Haplosporidium pinnae* [7,8]. Since 2019, *P. nobilis* has been listed as Critically Endangered on the IUCN Red List of Threatened Species "due to the drastic population size reduction caused by the still ongoing mass mortality event and the fact that the causing pathogen is still present in the environment" [9]. As a result of the significant conservation attention it has received, *P. nobilis* is now regarded as a flagship species [10]. This recognition not only highlights the importance of conserving *P. nobilis* but also raises awareness of broader marine environmental issues [10,11].

1.2. DNA Extraction in Conservation Biology

The current conservation concerns surrounding *P. nobilis* have prompted genetic studies to further investigate the population genetics of the species. Developing an effective protocol for DNA extraction is crucial for these efforts. Such a protocol should meet three primary objectives: (i) ensure the highest quality and yield of DNA for genetic studies, (ii) be simple and highly reproducible, and (iii) minimize damage to the sample species [12]. Removing inhibitors and contaminants is critical to obtaining high-quality animal DNA samples. In conservation biology, extracting high-quality DNA from old, degraded, and/or minute samples is a recurrent problem. In recent years, several new genomic tools, techniques, and strategies have been used in conservation studies. These new strategies simplify and speed up conservation research programs. However, a key challenge to these advances has been the improvement of DNA extraction methods with a focus on the characterization and quantification of amplifiable DNA, as it is the initial and critical step in downstream technologies [13].

Using shells as starting material for DNA extraction poses several methodological challenges. Shells typically contain very low amounts of genetic material due to their predominantly mineral composition, resulting in poor DNA yields and quality. Additionally, the calcified matrix of shells often requires harsh extraction conditions, which may further degrade the DNA. Moreover, shells frequently harbor PCR-inhibitory substances such as calcium carbonate and other minerals, complicating downstream analyses and leading to inconsistent amplification results. Addressing these issues by optimizing extraction protocols specifically tailored to shells is essential for enhancing the reliability and efficiency of genetic analyses based on this type of material [14–16].

The optimization of DNA extraction methods for specific target species is essential, and several standardized methodologies developed for other marine organisms have been applied to *P. nobilis* with varying degrees of success [3,17–21]. The selection of an appropriate DNA extraction method depends on multiple factors, including the available human and economic resources, laboratory materials and equipment, the type of environmental samples, and the target species. In conservation programs, particularly for *P. nobilis*, non-invasive sampling is essential, as the collected samples are often minute, dead, and/or highly degraded. At the same time, the extracted DNA must be of high quality and free of contaminants to ensure the success of downstream applications.

1.3. COI Marker and Species Identification

The mitochondrial cytochrome c oxidase subunit I (*COI*) gene has been adopted by the Consortium for the Barcode of Life (CBOL) as the standard genetic marker for DNA barcoding of individual animal specimens. This selection is largely attributed to the gene's compatibility with existing biodiversity databases, facilitating straightforward comparisons between newly generated sequences and previously documented data [22,23]. Such comparative analyses are critical for validating the reliability of *COI* as a barcode marker, thereby promoting its broader integration into global biodiversity initiatives [24,25].

The *COI* gene exhibits high levels of interspecific variability, making it particularly effective for species identification. Its broad applicability across diverse ecosystems—including marine, terrestrial, and freshwater habitats—further strengthens its value as a DNA barcoding tool. Additionally, *COI* sequencing methods are relatively rapid and cost-effective, enhancing accessibility for various research applications. *COI* sequencing is especially beneficial for detecting cryptic species, mapping their distribution and diversity, and providing clearer taxonomic differentiation when morphological identification is challenging. Consequently, a segment of approximately 650–700 base pairs within the *COI* gene has become widely established as the standard locus for animal DNA barcoding [25,26].

In the current study, we evaluated various DNA extraction protocols and PCR assays to identify the optimal method for *P. nobilis* identification. Alongside selecting the appropriate DNA extraction protocol, the choice of PCR chemistry, cycling conditions, and primers is equally important for assay optimization [27,28]. Overall, the DNA extraction and downstream analysis workflow involves numerous considerations, from selecting the most effective extraction protocol to developing species-specific assays for conservation purposes. Herein, we used only shell samples, either air-dried (dead individuals) or preserved in 96–100% ethanol (sample taken with a minimal destructive way from live individuals), to determine the protocol that yielded the highest-quality DNA for PCR analysis with robust results. The extraction protocols tested in this study included: Chelex-100, the sodium chloride (NaCl) method [29,30], a modified CTAB protocol [31], and the NucleoSpin Tissue Kit (Macherey Nagel GmbH & Co. KG, Düren, Germany). We explored variations in incubation times, temperatures, and shell sample preparation methods to optimize DNA yield and quality. Additionally, we assessed different cycling conditions and primer sets for the PCR assays, utilizing both nested and semi-nested approaches targeting the *COI* gene marker.

Our study introduces an optimal, time-efficient, and cost-effective DNA extraction protocol for obtaining high-quality *Pinna nobilis* DNA, essential for species identification, genetic monitoring, conservation efforts, health assessments, and recovery programs. DNA extraction from shells may be extremely valuable concerning any last surviving population, as well as, on the other hand, detecting habitats after mortality events or discriminating *P. nobilis* from the sister species *P. rudis*. Furthermore, we evaluated different PCR protocols and alternative sets of primers towards the optimum identification of *Pinna nobilis* using non-invasive methods.

2. Materials and Methods

Sample Collection and Material Preparation for DNA Extractions

In this study, we analyzed ten (10) *Pinna nobilis* individuals collected as described in [3,18]. To minimize disturbance and damage to the live individuals, only a minute section (3–5 mm) of the outer top part of the shell was carefully cut, on site, with scissors and placed in a plastic vial containing seawater. After the dive, all samples were immediately transferred to an Eppendorf microcentrifuge tubes filled with 96–100% ethanol.

Additionally, four dried samples from dead individuals collected during the dive were also used in the analysis.

Protocol 1 (P1): Chelex-100

Chelex-100 resin is a copolymer of styrene and divinylbenzene that is employed to chelate metal ions, which serve as cofactors for DNases, through its iminodiacetic acid groups. It was patented in 2011, by Xiong Hui, Xie Liqun, and Chen [32]. Chelex resin is utilized to chelate metal ions that function as cofactors for DNases through its iminodiacetic acid groups and stabilizes samples for downstream PCR applications. It is widely used because it allows for extractions to be conducted at room temperature. The Chelex beads bind cellular materials, leaving the DNA in the supernatant [33,34].

Duration: total time: 3 h (hands-on time: 20 min; incubation time 2.5 h).

1. Dilute 5 g Chelex-100 in 50 mL ddH_2O (always use freshly made solution).
2. Place 10% Chelex-100 solution (diluted in ddH_2O) on a magnetic stirrer.
3. *If the starting material is stored in ethanol, rehydrate the samples gradually by immersing them for 5 min each in the following ethanol (EtOH, 96–100%) and phosphate-buffered saline (PBS, 1%) solutions: (1) 75% EtOH/25% PBS, (2) 50% EtOH/50% PBS, (3) 25% EtOH/75% PBS, and (4) 100% PBS.*
4. Add 100 µL of well-shaken 10% Chelex-100 in your sample.
5. Add 10 µL of proteinase K (20 mg/mL), stored at −20 °C and thawed on ice, to each tube.
6. Vortex tubes for 15 s.
7. Incubate for 1 h in 56 °C.
8. Incubate in a PCR machine at 95 °C for 30 min.
9. Centrifuge tubes at 4000 rpm for 3 min.
10. Incubate in a PCR machine at 95 °C for 30 min.
11. Centrifuge tubes at 4000 rpm for 3 min.
12. Transfer 50 µL of the supernatant to a new tube.

NB1: The protocol was additionally evaluated without proteinase K, yielding less successful outcomes.

NB2: Hydrating the ethanol-stored shells in PBS significantly improved the DNA extraction.

NB3: Various incubation times with proteinase K ranging from 1 h to 12 h (overnight) were tested, with no observed differences in the quality of the DNA extracted.

Protocol 2 (P2), NaCl Precipitation, modified from [24,25].

The salting-out method has been reported to produce high-quality DNA, while it is relatively time-efficient, cost-effective, and, importantly, does not utilize toxic reagents.

Duration: total time: 18 h (hands-on time 2.5 h; incubation time: 15 h).

Salt solution (6 M NaCl) was used to remove cellular protein and concentrate genomic DNA. Isopropanol was used for desalting and DNA precipitation.

1. If the starting material is stored in ethanol, rehydrate the samples gradually by immersing them for 5 min each in the following ethanol (EtOH, 96–100%) and phosphate-buffered saline (PBS, 1%) solutions: (1) 75% EtOH/25% PBS, (2) 50% EtOH/50% PBS, (3) 25% EtOH/75% PBS, and (4) 100% PBS.
2. Add 87.5 µL of 10% SDS solution to each tube.
3. Add 700 µL of Extraction Buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2) to each tube.
4. Add 10 µL of proteinase K (20 mg/mL), stored at −20 °C and thawed on ice, to each tube. Carefully pipette.
5. Briefly vortex to mix well.
6. Incubate the samples at 56 °C in a water bath overnight. Occasionally vortex briefly or use a shaker water bath.

7. Add 300 µL of saturated NaCl solution (6 M) to each tube and place them on a rocker for 5 min. Leave on ice for 10 min.
8. Centrifuge at maximum speed for 30 min.
9. Carefully transfer 700 µL of the supernatant to a new 2 mL Eppendorf tube using a pipette.
10. Add an equal volume of ice-cold isopropanol or 100% ethanol to each tube and invert 20–30 times to mix well.
11. Leave the tubes at −20 °C for 30 min.
12. Centrifuge at maximum speed for 20 min to pellet the DNA.
13. Immediately after centrifugation, carefully remove the supernatant with a pipette without losing the DNA pellet.
14. Add 800 µL of 70% ice-cold ethanol to each tube and leave at room temperature for 15 min.
15. Centrifuge at maximum speed for 10 min and carefully remove all the ethanol with a pipette without losing the DNA pellet.
16. Repeat the previous step.
17. Dry the DNA pellet at 37 °C for 10–15 min by placing the tubes with open caps on a heat block to evaporate all the ethanol. Important: check by lightly flicking the tubes for any ethanol residues. There should be no droplets on the walls.
18. Resuspend the DNA in 30 µL of ultrapure water by pipetting 5–10 times and leave the samples for 10 min at room temperature. Store the samples at −20 °C.

Protocol 3: fast-CTAB (cetyl trimethyl ammonium bromide) modified from [31]

CTAB is a cationic detergent used initially to extract DNA from plant tissues because it effectively precipitates polysaccharides and is especially effective in eliminating interfering secondary metabolites [31]. The CTAB DNA extraction protocol has been modified numerous times, and its use has expanded to successfully extract DNA from various species and difficult tissues. Isolating high-quality DNA free from polysaccharides, secondary metabolites, or other PCR-inhibiting compounds is essential for successful downstream applications like PCR, amplification, cloning, and sequencing.

Duration: total time: 3.5 h (hands-on time: 2.5 h; incubation: 1 h).

We used a simplified protocol of [31] omitting the use of b-mercaptoethanol and proteinase K and the initial step of PBS rehydration of the material.

1. If the starting material is stored in ethanol, take it out and leave it on a paper towel for 15 min or wash it in water to wash out the ethanol.
2. Transfer the shell material to a 1.5 mL tube.
3. Add 200 µL CTAB and grind with a plastic pestle.
4. Vortex for a few secs.
5. Incubate at 55–60 °C for 1 h (can be extended to overnight, if convenient).
6. Add an equal volume (here 200 µL) of chloroform (24:1 isoamylic alcohol used).
7. Mix phases by inverting tubes many times (1–2 min).
8. Centrifuge at 8000 rpm for 5 min.
9. Transfer the upper, aquatic phase to a new 1,5 mL tube. Avoid debris and organic phase.
10. Add equal volume of isopropanol (here: 150–200 µL) and invert tubes several times to mix.
11. Place at −20 °C for 20 min (can be extended to overnight, if convenient).
12. Centrifuge at maximum speed (13,000 rpm) for 15–20 min.
13. Discard supernatant.
14. Add 500 µL of 70% ethanol and invert tubes 5–6 times.
15. Centrifuge at maximum speed (13000 rpm) for 5 min.
16. Discard supernatant.

17. Let it dry at 37 °C (or in R.T. overnight).
18. Dilute in 50 µL of ultrapure water.

Protocol 4 (P4), spin column protocol [32] **using the Nucleospin Tissue (Macherey-Nagel)**

Silica column-based DNA extraction kits operate on the principle that DNA binds to the silica membrane under high salt and low pH conditions, while impurities such as proteins, lipids, and polysaccharides are washed away using ethanol-based buffers. Pure DNA is then eluted with a low-salt buffer or water [35,36]. These kits offer high purity and yield, producing DNA free of contaminants and suitable for sensitive downstream applications like PCR, qPCR, sequencing, and cloning. The process is fast and convenient, typically taking 30–60 min with minimal hands-on time. Standardized reagents and protocols ensure consistent results across samples, and the use of non-toxic buffers makes these kits safe for beginners and non-experts.

Duration: total time: 12 h 30 min (hands-on time: 1 h 30 min; incubation time: 12 h).

We followed the manufacturer's instructions with the following options:

1. Shells were submerged in 500 µL of PBS 1% solution 1% to hydrate (2 h).
2. Extension of incubation time with proteinase K overnight.
3. At the final elution step, we used 50 µL of prewarmed Buffer BE (70 °C) and incubated for 10 min before centrifugation (1 min at 11,000× *g*).

NB1: Shorter incubation times (1 h and 3 h) were tested with no positive results.

NB2: Hydrating the shells in PBS significantly improved the DNA extraction.

For all protocols, we used 1.5% agarose gel electrophoresis to qualitatively and semi-quantitatively assess the length of DNA fragments in our extracts, using 5 µL of extracted DNA per sample. Given the nature of the samples (degraded from dried individuals) and the potential presence of contaminants (e.g., DNA from other organisms on the shell fragments), we opted against spectrophotometric quantification. This method, which relies on absorbance at 260 nm, lacks specificity in distinguishing intact from fragmented DNA and is susceptible to interference from RNA, proteins, and other contaminants, potentially leading to overestimated concentrations and unreliable purity assessments.

To ensure the integrity of the extracted DNA, we instead performed PCR testing and sequencing. All samples underwent various PCR assays with different cycling conditions and primer sets, including nested and semi-nested approaches, to evaluate DNA quality and optimize amplification efficiency. PCRs targeted a fragment of the *COI* gene, and successfully amplified products were sent for bidirectional sequencing to confirm target DNA identity and mitigate the risk of misleading quantification due to degradation or contamination [14].

Initial PCR amplifications were carried out using standard primer pair LCO1490 and HCO2198 [37]. Specifically, after isolation, 1 µL DNA was used as a template in PCR with LCO1490-HCO2198 primers to amplify a fragment of the cytochrome oxidase subunit I (*COI*) gene. The PCR mixture included 1x KAPA2G Fast Multiplex PCR Mix (Kapa Biosystems, Wilmington, MA, USA), 10 µM of each primer, and approximately 10 ng of genomic DNA (or PCR product for the semi-nested/nested PCR), with the volume adjusted to 25 µL using double-distilled water (ddH_2O). A gradient protocol was initially employed to determine the optimal annealing temperature for the primers used. The thermocycling conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 37 cycles of 94 °C for 30 s, 45–58 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

Due to suboptimal and contaminated sequencing results characterized by weak signal intensity, overlapping peaks, and ambiguous base calls (see Results), novel internal primers targeting the *COI* fragment (PnCOI_F_MK22 and PnCOI_R_MK22) were designed [18] using Primer-Blast from GenBank https://www.ncbi.nlm.nih.gov/tools/primer-blast/

(accessed on 1 May 2022). Additionally, two other primer sets previously suggested in the literature were also tested [17,38]. A semi-nested PCR approach was subsequently adopted; 1 μL of DNA from the best initial PCR with LCO1490 and HCO2198 primers was used as a template for a second amplification round using primer combinations of forward PnCOI_F_MK22 and reverse primers from each primer pair. The efficacy of primers PnCOI_F_MK22 and PnCOI_R_MK22 was further evaluated through nested PCR protocols. All primers utilized in this study and their specific combinations in the PCR assays are summarized in Tables 1 and 2, respectively.

Table 1. Primers used for the amplification of *COI* gene fragment at *Pinna* sp. samples [14].

Primer Name	Primer Sequence (5′–3′)	Reference
LCO1490 HCO2198	GGTCAACAAATCATAAAGATATTGG TAAACTTXAGGGTGACCAAAAAATCA	[37]
PnCOI_F_KTSR08 PnCOI_R_KTSR08	CCCTGCCAAATTACACCAGT TTTTGGCTTTTGCCTTCTTC	[17]
PnCOI_Fdg_KTSR08 PnCOI_Rdg_KTSR08	CCCTAGCCAAAATTACACCAGT GAAGAAGGCAAWAGCCAAAA	[17]
PnCOI_F_MK22 PnCOI_R_MK22	CAACACAGGAAGAGAGACTACCA GGCAGGGTTTTTGGGGGA	[18]
PnCOI_L_SAN13 PnCOI_H_SAN13	GGTTGAACTATHTATCCNCC GAAATCATYCCAAAAGC	[38]
Pmur_COIF_KTSR08 Pmur_COIR_KTSR08	GAAAGTGCCCGGTAACAAAA TGATAGGGGTTCCGGATATG	[17]
Pmur_COIFdg_KTSR08 Pmur_COIRdg_KTSR08	GAAAGTGCCCRGTWACAAART CATATCYGGMACCCCTATCA	[17]

Table 2. Combination of primers used for the semi nested and nested amplification of *COI* gene fragment at *Pinna* sp. samples.

PCR Trial	1st PCR Primer Pair	2nd PCR Primer Pair	Amplicon Size
a: 1st semi-nested	PnCOI_F_KTSR08- PnCOI_R_KTSR08	PnCOI_F_MK22- PnCOI_R_KTSR08	ca. 500 bp
b: 2nd semi-nested	PnCOI_Fdg_KTSR08- PnCOI_Rdg_KTSR08	PnCOI_F_MK22- PnCOI_Rdg_KTSR08	ca. 550 bp
c: 3rd semi-nested	PnCOI_L_SAN13- PnCOI_H_SAN13	PnCOI_F_MK22- PnCOI_H_SAN13	ca. 490 bp
d: 4th semi-nested	Pmur_COIFdg_KTSR08- Pmur_COIR_KTSR08	PnCOI_F_MK22- Pmur_COIR_KTSR08	ca. 650 bp
e: nested	LCO1490-HCO2198	PnCOI_F_MK22 -R_MK22	ca. 465 bp

3. Results

DNA Extraction, PCR Amplification, and Sanger Sequencing

All DNA extraction methods returned positive results for all samples. Chelex-100 and NaCl protocols showed less degradation of DNA (decreased intensity of the smears). However, Nucleospin Tissue kit and CTAB methods appeared to result consistently in higher molecular weight of DNA across all samples. It should be noted that samples numbered 1 and 10 originated from dead shell material.

Regardless of the DNA extraction method used (Figure 1), we had positive amplification results using the LCO1490 - HCO2198 primers [37] for most of the samples

(Figure 2). However, PCR results when using the DNA extracted with NaCl and CTAB protocol appeared to be more consistent across samples. Specifically, *COI* amplification had an average success rate of 30% using DNA extracted from Chelex. We proceeded with Sanger sequencing using forward and reverse primers (LCO1490 + HCO2198). Surprisingly, the sequencing results were not optimal and appeared contaminated in all cases (Figure 3), hindering the correct and reliable identification at the species level. The chromatogram displays poor-quality sequencing data, characterized by weak signal intensity, overlapping peaks, and ambiguous base calls. These features indicate the failure of the PCR amplification step, degraded template DNA, or insufficient reaction optimization. The absence of distinct, well-resolved peaks further confirms the lack of specific and efficient amplification of the target region. For subsequent nested and semi-nested PCR approaches, the initial PCR products obtained from NaCl and CTAB extractions (Figure 2) were used as templates, as these exhibited greater consistency across samples. Comparing results between semi-nested and nested PCRs revealed superior amplification quality with the nested PCR approach (Figure 4). Nested PCR products were successfully sequenced (bidirectionally), and the chromatograms (Figure 5) were significantly improved, allowing for the reliable identification of the species using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 30 October 2024). All sequences generated in this study have been deposited in GenBank under accession numbers: OR479010-OR479025 [18].

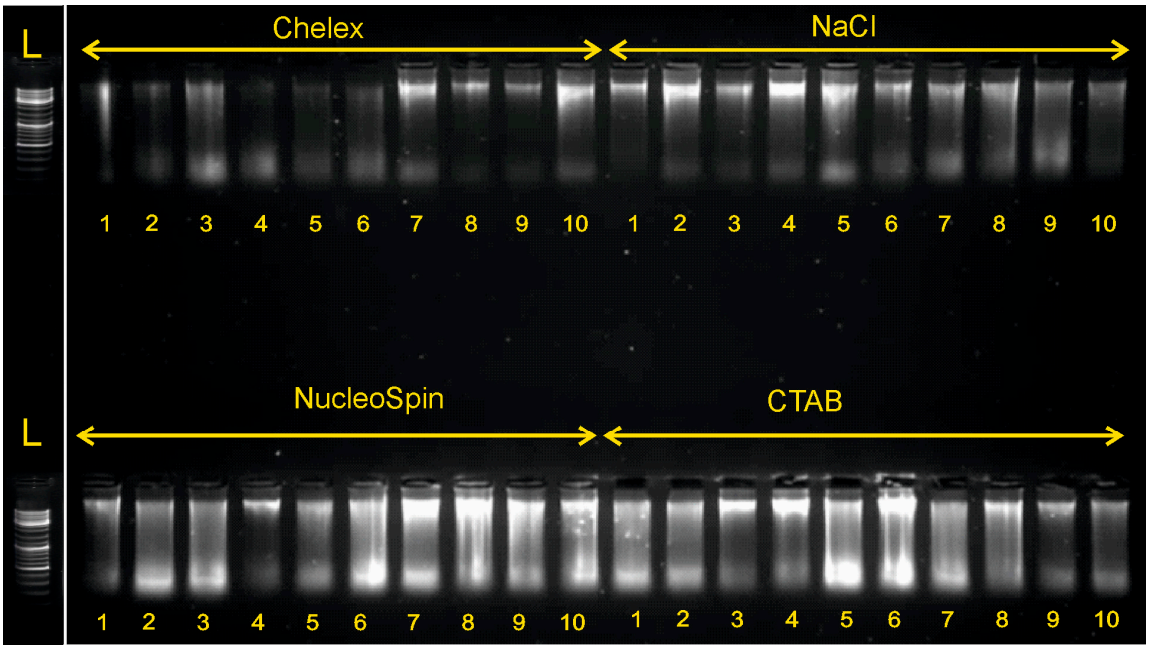

Figure 1. The qualitative and semi-quantitative analysis of genomic DNA indicated that the CTAB protocol and Nucleospin kit were the most effective for extracting high-molecular-weight DNA across the samples. However, the NaCl protocol showed less DNA degradation. Chelex-100 extraction results were not consistent amongst samples. **L**: 100 bp DNA Ladder (New England Biolabs, Ipswich, MA, USA).

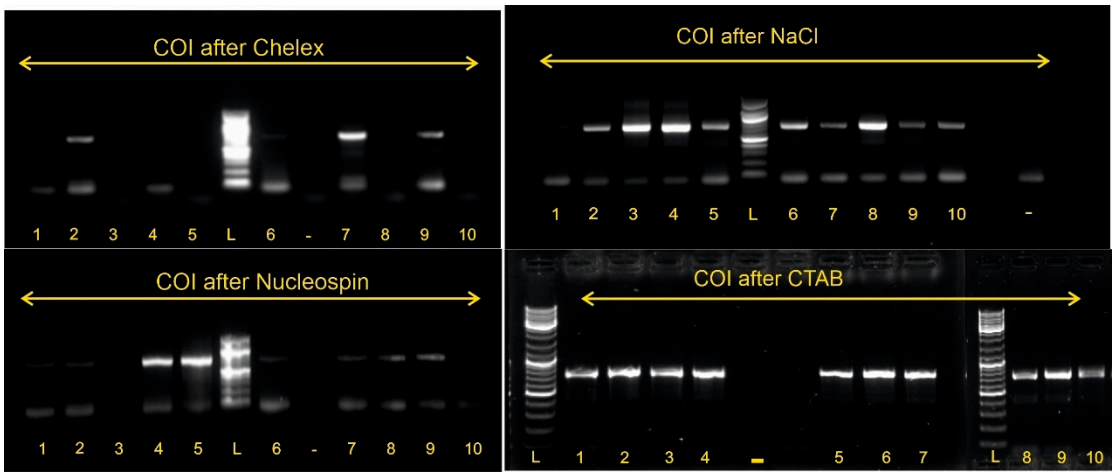

Figure 2. Image is a composite of four photo gel electrophoresis depicted results after the PCR amplification using the Folmer et al. primers [37]. **L**: 100 bp DNA Ladder (New England Biolabs, Ipswich, MA, USA). Samples are presented in the same order as shown previously in Figure 1 depicting DNA extraction.

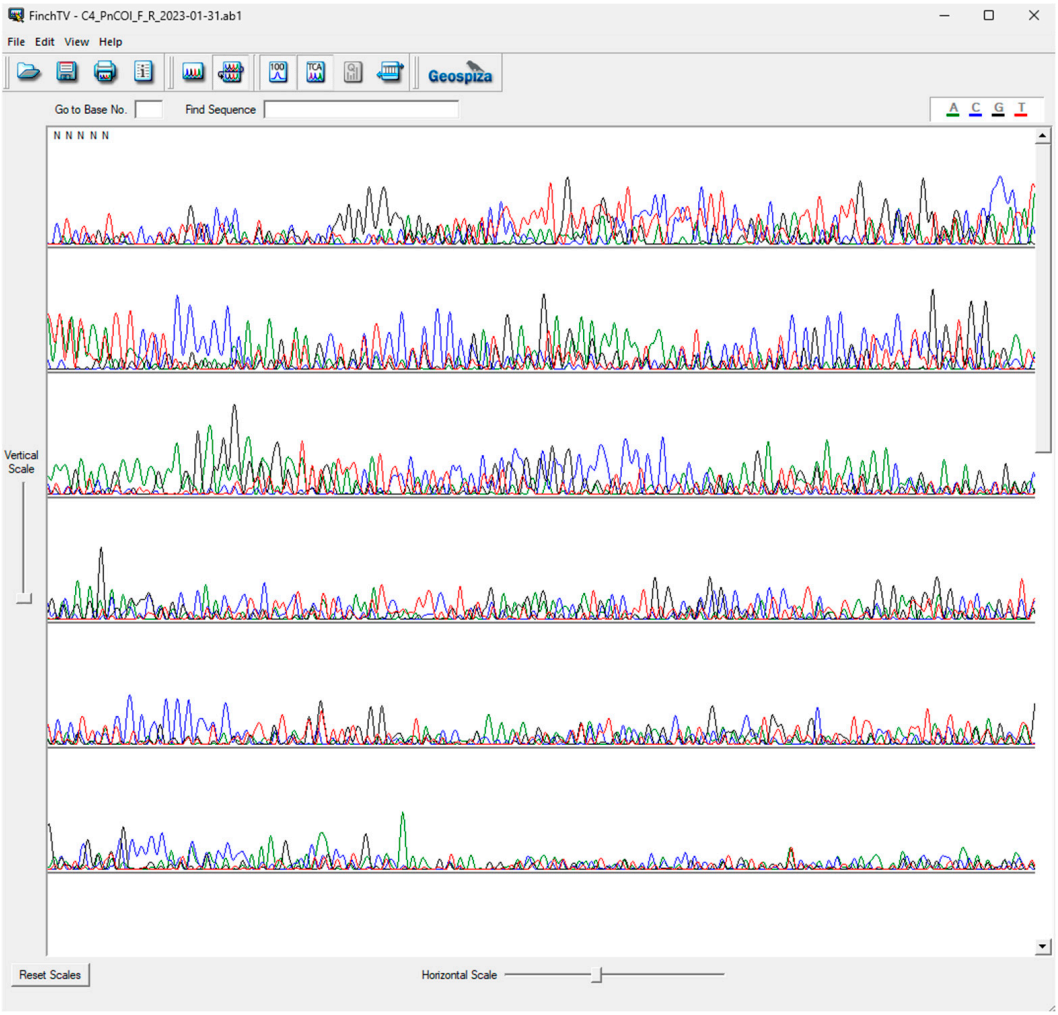

Figure 3. Chromatogram indicative of poor sequencing results of PCR using LCO1490-HCO2198 [30] primers.

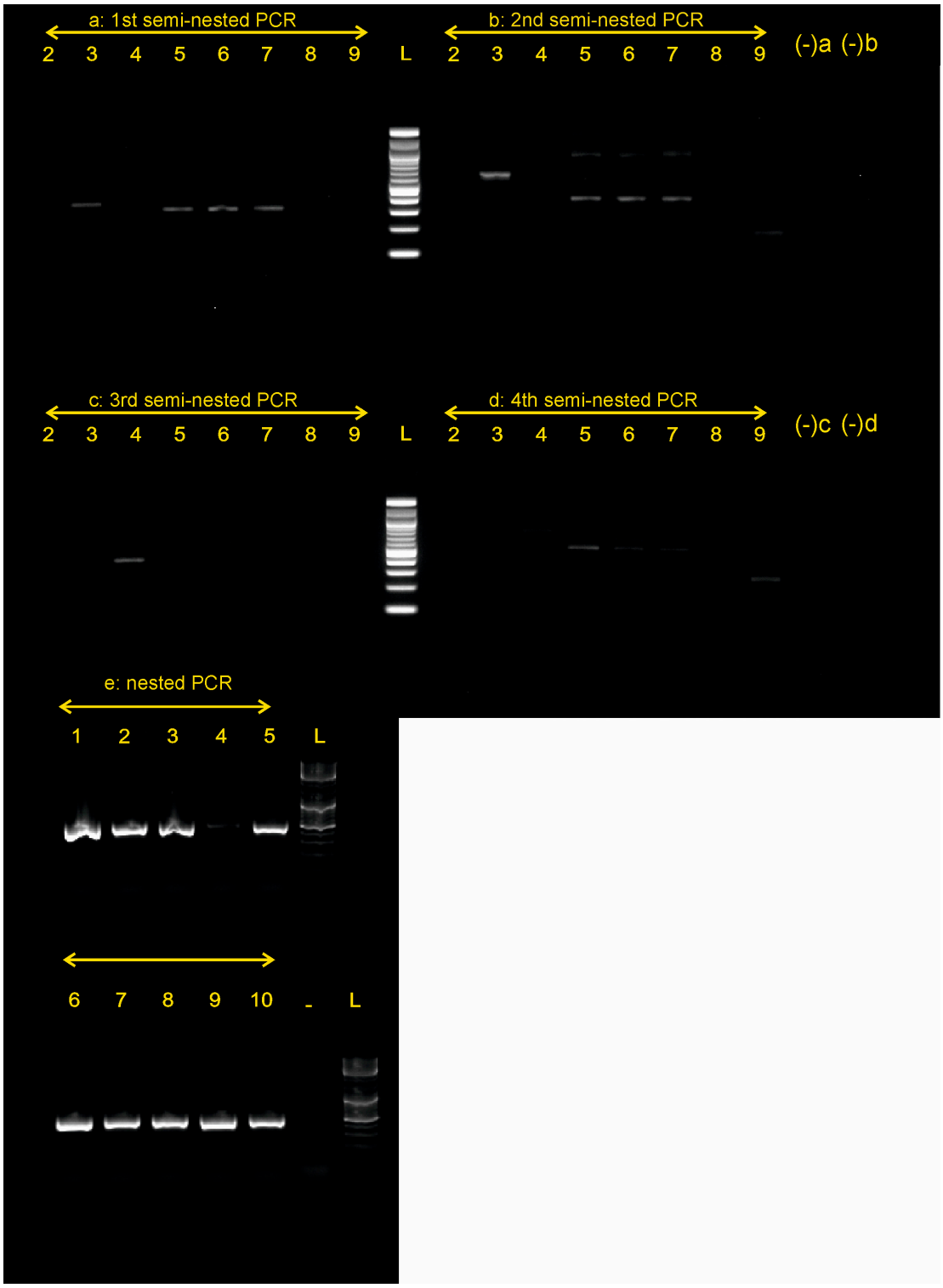

Figure 4. (**a**–**d**): The image is a composite of two gel electrophoresis. It depicts semi-nested PCR results of *COI* after CTAB extraction method using different sets of primers. The same results are derived when using the extracted DNA with the NaCl method. (**e**) Example of nested PCR for *COI* after the CTAB method. The same results are returned when using the DNA extracted with NaCl protocol. L: 100 bp DNA Ladder (New England Biolabs, Ipswich, MA, USA). The primers used in every trial are summarized in Table 2. Numbers represent samples in the same order as shown previously in Figures 1 and 2.

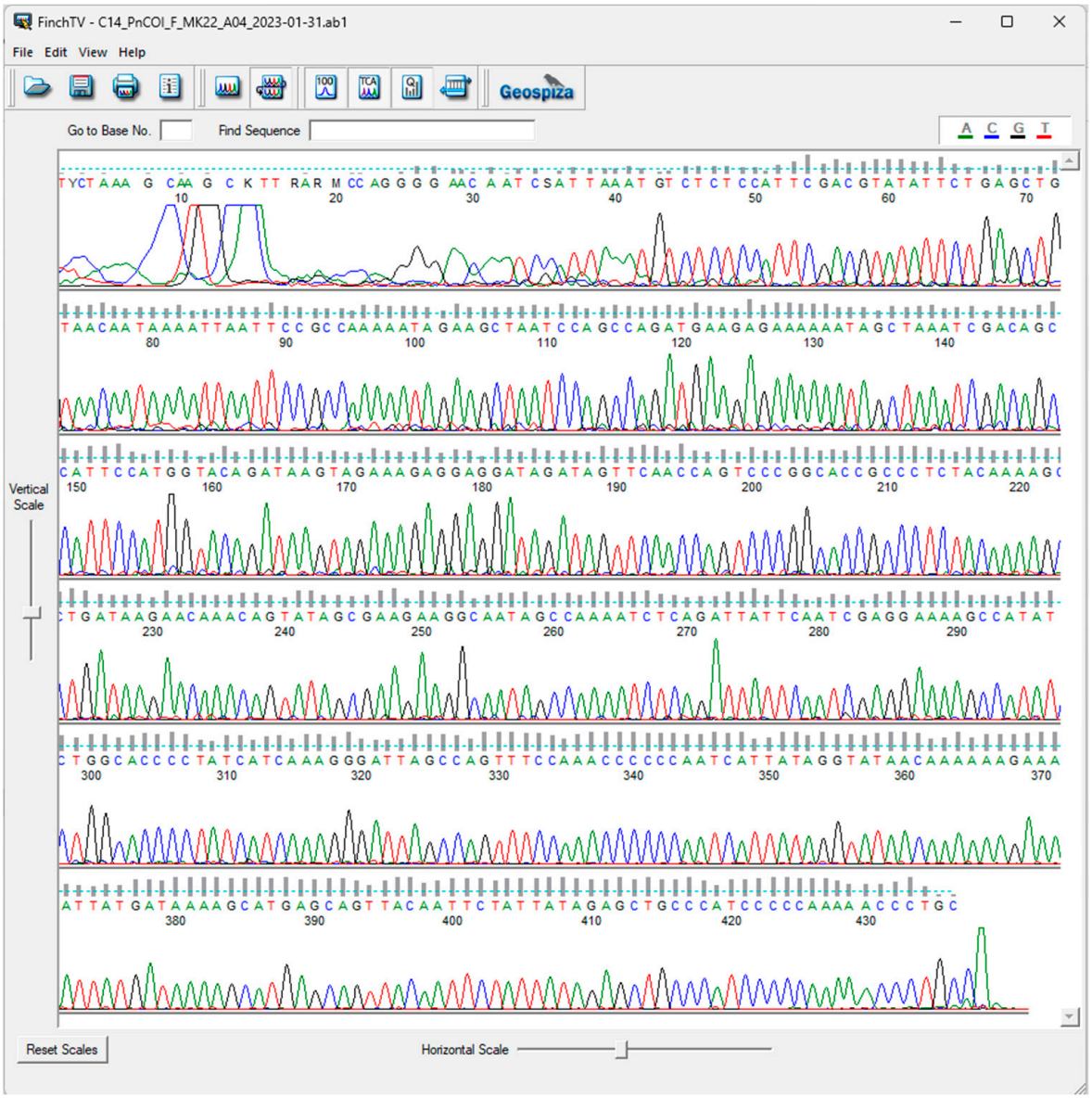

Figure 5. Indicative chromatogram of a good sequencing result of PCR using the nested approach with PnCOI_F_MK22 +R_MK22 primers.

4. Discussion

Molecular genetic techniques have revolutionized the study of genetic diversity, population structure, and phylogenetic relationships in endangered species. Several factors have been reported to influence the reliability and repeatability of genetic analyses, highlighting the importance of maximizing the accuracy of DNA isolation [12,39,40].

In the present study, we aimed to evaluate different DNA extraction methods both quantitatively and qualitatively, as insufficient DNA yield or quality could result in loss or degradation of specific DNA and eventually compromise genetic analysis in endangered species (Table 3). Additionally, we assessed different primer sets for the downstream molecular genetic analysis and identification of *P. nobilis*.

Table 3. Quantitative comparison of DNA extraction protocols' PCR amplification and Sanger sequencing success rates. Primers used for each case are analyzed in Table 2.

DNA Extraction Method	**Chelex-100**	**NaCl**	**Fast-CTAB**	**NucleoSpin**		
Positive indication of high-molecular-weight DNA %	50.0	100.0	100.0	100.0		
Consistency *	1	2	3	3		
	***COI* amplification success rate %**				**Sanger Seq success rate on successful PCRs% after CTAB**	**Total successful taxonomic identification of samples % after CTAB**
DNA extraction method	**Chelex-100**	**NaCl**	**Fast-CTAB**	**NucleoSpin**		
LCO-HCO	30.0	90.0	100.0	80.0	0	0
1st semi-nested PCR		50.0	50.0		75	30.0
2nd semi-nested PCR		37.5	37.5		100	30.0
3rd semi-nested PCR		12.5	12.5		100	10.0
4th semi-nested PCR		0.0	0.0		100	0.0
Nested PCR		100.0	100.0		100	100.0

Note: *: 1 = low, 2 = medium, 3 = high.

4.1. DNA Extraction Protocols: Comparing Yield, Cost, and Time Efficiency

Analyzing the cost and time efficiency of the evaluated DNA extraction protocols is a key factor when selecting the most suitable method. The major bottleneck in rapid DNA isolation is usually the higher cost of single spin columns compared to other protocols. In most established protocols, tissues and cells as starting materials are abundant, but herein there is a need for an optimized protocol using a minute shell fragment collected with minimal disturbance from live *Pinna nobilis* individuals. Our results on the comparison of the different protocols evaluated are summarized in Table 4.

Table 4. Comparison of Chelex-100 resin, NaCl precipitation, fast-CTAB, and commercial kit for DNA extraction in terms of effectiveness, time required, and cost per sample.

Protocol	**Effectiveness (Purity and Yield)**	**Hands-On Time Required**	**Cost Per SAMPLE**
Chelex100	Moderate: Yields DNA suitable for PCR but may not be ideal for applications requiring high-purity DNA.	Very low: Quick, single-tube process (~20–30 min).	Very low: Inexpensive reagents; ideal for high-throughput and routine PCR applications.
NaCl Precipitation	High for routine DNA extractions; yields clean DNA but may not efficiently remove all contaminants (e.g., proteins).	Moderate: labor-intensive with multiple centrifugation and incubation steps and washing (~2–3 h and an overnight step).	Low: Requires minimal reagents and equipment; very economical for bulk extractions.
Fast-CTAB	High: effective for tissues with polysaccharides and ideal for minute starting material; adaptable for diverse samples.	Moderate: Several steps involving incubation, precipitation, and washing (~2–3 h).	Low: Requires basic reagents and equipment; minimal recurring costs.
Silica-based column (NucleoSpin Tissue)	High: Produces high-purity DNA suitable for sensitive downstream applications (e.g., qPCR, sequencing), user-friendly for non-experts.	Low to moderate: Rapid, streamlined process (1–2 h and an overnight incubation step)	High: Commercial kits are expensive; cost scales with sample volume and kit usage.

One of the most time-consuming steps in DNA extraction is the lysis or tissue digestion phase. Traditional methods rely on protease activity at high temperatures and require reagents that are toxic and dangerous to humans, lab personnel, and the environment. Any activity that minimizes the concentration of toxic substances in the laboratory is highly recommended regarding the reuse of waste and waste management [41]. Efficient DNA extraction is dependent on multiple technical factors, one of which is the application of a suitable method for primary lysis or digestion procedure. Therefore, it is important to efficiently isolate DNA using both traditional and newly implemented methods designed to lower the cost and enable high-throughput analysis. The selection of an appropriate method is generally based on the research objectives and the number of samples to be examined, in addition to possible time and cost limitations. The aim of this study was to compare the cost effectiveness and time for five different DNA extraction methods using the same types of samples.

Chelex-100: The Chelex-100 extraction method (patented in 2011 by Xiong Hui, Xie Liqun, and Chen Jiayi) [42] is an economical and efficient approach for processing large numbers of samples quickly. The Chelex-100 resin effectively inhibits DNases that remain active even after boiling. This protocol is highly suitable for DNA barcoding and can potentially be applied to shotgun sequencing. Moreover, performing extractions in a 96-well plate format enables the processing of 96 samples in under an hour, if there is no prior incubation step needed [43]. Chelex-100 is a highly efficient chelating resin widely utilized in nucleic acid extraction and purification due to its ability to facilitate extractions at room temperature while preserving DNA in a relatively contaminant-free solution. The resin functions by selectively removing positively charged ions, including heavy metals, which helps protect DNA from nuclease activity that could otherwise lead to its degradation. The Chelex-100 method is less effective at DNA recovery than other methods, but it provides good quality DNA and is fast and easy to use. Due to the cell lysis process, it could generate noise, and for this reason, it may not be the most appropriate method when employing different concentrations of tissue and/or challenging starting material. In our study, we did not have a positive *COI* sequencing result for all individuals processed with this method. The protocol seems not appropriate for minute and degraded starting shell material as in the case of this study.

NaCl precipitation method: The NaCl method [29,30] has been demonstrated to produce high-quality DNA. It offers notable advantages, including lower cost, and the use of non-toxic reagents. This method is also versatile, being applicable for DNA extraction from blood, suspension cultures, and tissue homogenates. Main problems with this method: Sometimes salts precipitate trapping proteins close to the DNA, particularly when processing large amounts of tissue. These proteins could be co-precipitated with the DNA, affecting its purity. DNA purity could be good if there are no properly solved proteins. The addition of 70% EtOH wash can also guarantee that these proteins do not influence purification. After re-dissolving the DNA, it can be used for PCR or other molecular biology techniques, just like DNA extracted using any other commercial kit. Also, caution in handling concentrated NaCl is required as it can be dangerous to skin exposure. With careful optimization, specifically concerning the shell-to-reagent ratio and incorporation of ethanol washes, the NaCl precipitation method is recommended for DNA extraction from *Pinna nobilis* shell fragments.

Fast-CTAB: This protocol is recognized for its effectiveness in producing pure DNA suitable for downstream applications such as PCR, qPCR, and next-generation sequencing. CTAB extraction typically yields high-quality DNA with minimal contamination from proteins and polysaccharides, critical for reliable performance in molecular analyses. The protocol uses readily available and relatively inexpensive reagents, making it a cost-effective

choice for laboratories with limited resources. The CTAB method can be quite versatile, increasing its utility across different research areas. However, the use of chloroform is a limitation of the method because of its volatility and toxicity and the subsequent handing requirements. The second major limitation of the original protocol by [31] is its labor-intensive and time-consuming aspects; however, in this study, we suggest a "fast-CTAB" method **that significantly reduces the time needed for sample processing** with excellent results regarding DNA yield and successful downstream analysis. CTAB protocols with different modifications have consistently resulted in high DNA yields from challenging sample material [44,45].

Nucleospin Tissue kit (Macherey Nagel GmbH & Co. KG, Düren, Germany): Spin column-based DNA extraction methods [32] have multiple advantages; hence, they have become a standard technique for isolating DNA in molecular biology laboratories due to their simplicity, efficiency, and reliability. These methods provide a streamlined workflow and that relies on the selective binding of DNA to a silica membrane under chaotropic salt conditions, followed by a series of washing and elution steps. While spin column-based techniques are widely used for their convenience, their performance and practicality depend on the specific requirements of the application. One limitation is that they may not recover as much DNA as traditional methods, particularly from samples with extremely low DNA content. This can be a limitation when analyzing rare or degraded specimens. The silica membrane may preferentially bind certain DNA fragment sizes, potentially affecting the recovery of small or degraded DNA, which is common in biodiversity samples such as environmental DNA (eDNA). The NucleoSpin Tissue Kit has been employed in other molluscan DNA extraction studies, particularly involving shell material. For instance, Geist et al. (2008) [14] used this kit to extract DNA from the shells of the freshwater pearl mussel (*Margaritifera margaritifera*). They modified the standard protocol by doubling the volumes of lysis buffer and ethanol to enhance DNA yield. Despite these adjustments, the study found that phenol–chloroform extraction methods outperformed the NucleoSpin Tissue Kit in terms of DNA quantity and quality from shell samples. Other studies have also reported on silica-based protocols to be suboptimal when dealing with shell material [16,45–47].

Emerging technologies, such as paper-based extraction techniques or direct lysis methods, aim to provide cost-effective and rapid solutions without significantly compromising DNA quality [48]. Direct lysis methods are particularly advantageous for field applications or resource-limited settings, where infrastructure for traditional extraction may be unavailable. However, these methods often yield DNA of variable purity, potentially impacting downstream applications such as PCR or sequencing. Cost analysis studies consistently emphasize the importance of aligning technique selection with experimental goals. High-fidelity applications, such as next-generation sequencing, may justify higher upfront costs for methods yielding superior-quality DNA, while routine diagnostics may benefit from quicker and cheaper alternatives. Balancing cost and time efficiency thus requires an integrative approach, considering the scope of the project, sample throughput, and the quality of DNA required for downstream workflows [49].

4.2. Conventional vs. Nested PCR: Advantages, Limitations, and Enhancements with Specific Primers

Nested polymerase chain reaction (PCR) is a modification of conventional PCR designed to enhance specificity and sensitivity, particularly when the target DNA is present in low quantities or prone to non-specific amplification. Unlike standard PCR, which employs a single primer pair, nested PCR utilizes two successive amplification rounds with distinct primer sets. The first set amplifies a broader DNA region, while the second set, annealing within the initial amplicon, ensures a more selective and accurate amplification. This approach significantly reduces non-specific products and background noise, making

nested PCR highly effective for detecting rare genetic variants, degraded DNA, and low-abundance pathogens [50,51]. Compared to standard PCR, nested PCR offers superior sensitivity, as it can detect even low-copy-number DNA target. By incorporating a second amplification step, it minimizes false positives from initial contamination, provided that rigorous precautions are taken when handling primers in separate environments. However, its increased specificity and sensitivity come with certain limitations, including a higher risk of cross-contamination, longer processing times, and greater labor intensity due to additional primer design, reaction setup, and post-PCR handling.

Regarding primer efficiency, our results clearly indicate that universal primers and other primer sets from the literature performed poorly. The use of universal primers typically encounters challenges related to binding efficiency, stability, and primability across species, which becomes particularly problematic with degraded or low-concentration DNA samples [37,52]. Contrarily, our newly designed primers—especially when employed in nested PCR protocols—demonstrated significantly improved specificity and amplification efficiency. This outcome emphasizes the critical importance of meticulous primer design and optimization [53,54]. Specifically, primers should ideally exhibit perfect binding to target sequences to ensure reliable amplification, even under challenging conditions such as those involving minute samples or degraded DNA [55]. Our findings align with recommendations from previous studies that emphasize using overlapping short-amplicon primer sets to overcome limitations encountered with universal primers, especially when working with challenging samples [56,57] (Giribet et al., 2007; Dabney et al., 2013).

Our results corroborate that a nested PCR utilizing a combination of universal [37] (for the initial PCR) and specific [18] (for the nested PCR) newly designed primers can give excellent results with degraded and/or minute starting material. Without nesting, standard PCR is biased towards amplifying the fastest-replicating target sequence in the mixture, hence the inability to resolve a complex sample. Our primers' combination enhanced reaction efficiency, ensuring greater accuracy and reliability in species identification and genetic analysis [58]. By refining nested PCR with improved primer design, this contribution strengthens its application in biodiversity research, molecular diagnostics, and environmental DNA studies [51].

4.3. Pros and Cons of COI Gene as a Stand-Alone Marker for Species Identification

Within the context of single-gene approaches, the *COI* gene has several advantages as a genetic marker for species identification. Firstly, the *COI* gene is the most well-studied and widely used DNA marker for animals. Its high mutation rate, compared to other genes, is a significant advantage, as it enables the identification of closely related species that are morphologically indistinguishable [59]. Secondly, it is easy to obtain *COI* sequences from various biological samples, including tissue samples, excrement, alcohol-preserved specimens, and environmental DNA. This greatly facilitates the assessment of biodiversity, especially in poorly studied regions. In addition, the robustness of *COI* across different taxa makes it a versatile DNA marker. The *COI* gene is suitable for a wide range of organisms, from land and freshwater to marine environments [22]. In addition, juvenile and damaged specimens, which are often discarded in traditional identification work, can easily be identified at the DNA level. Moreover, the methodology of DNA barcoding using the *COI* gene as a taxonomic reference is cost-effective and simple, that can be performed with common laboratory equipment, making it accessible for widespread research and conservation initiatives. Finally, the successful amplification of the *COI* gene enables the addition of new species to the database resources for future taxonomic reference, which is also advantageous [60,61].

While the *COI* gene has several advantages as a stand-alone DNA marker, it is essential to consider its drawbacks. First of all, the use of the *COI* gene is considered more appropriate for animals but is limited for fungi and plants. The *COI* gene in fungi exhibits several undesirable features like amplification challenges and insufficient variability [62]. Regarding the plants, the *COI* barcode is ineffective for identification due to the slow evolution and low substitution rates of their mitochondrial genes [63]. In animals, *mtCOI* is maternally inherited and lacks recombination, which can lead to discrepancies in species identification due to phenomena such as introgression, hybridization, and incomplete lineage sorting [20,64–67]. Additionally, in several bivalves, doubly uniparental inheritance (DUI) of mtDNA and two mitogenome types have been observed [68], a fact that could impact the utility of this marker for this group of animals, but DUI has never been reported in *Pinna nobilis* [69]. The hypervariability of the *COI* gene in some taxa may also render it less reliable for phylogenetic inference. Rapidly diverging genes can result in long branch attraction artifacts, where closely related taxa are erroneously inferred as sister taxa [70–72]. Furthermore, high levels of molecular convergence have been observed in different taxa, where distantly related species share similar *COI* sequences. This convergence complicates accurate identification based on a single marker, as different geographical populations and species of the same family may cluster together [20,73]. Finally, the *COI* gene may have limited capacity to recognize intra-species diversity and may not provide sufficient resolution in cases of recent speciation or cryptic diversity. Therefore, caution should be exercised when interpreting the sequencing results of this gene.

Integrative taxonomy addresses these limitations by incorporating multiple lines of evidence, including nuclear DNA markers, morphological traits, ecological data, and behavioral characteristics, to achieve a more robust and unequivocal species delineation [74]. This approach enhances the accuracy of species delineation by overcoming the limitations of relying on a single marker. It has been successfully applied to uncover cryptic species and resolve taxonomic ambiguities, while also contributing to a deeper understanding of species distribution, biodiversity, and conservation planning [75]. Integrative taxonomy is particularly valuable in studying organisms in extreme environments, where collaboration and data sharing among researchers are essential for maximizing the utility of collected data. Therefore, while *COI* remains a powerful tool for DNA barcoding, its efficacy is significantly enhanced when combined with complementary molecular and morphological datasets within an integrative taxonomic framework [74,76,77].

5. Conclusions

The results presented herein confirm that the proposed methodology effectively overcomes challenges associated with genetic analysis of the endangered *Pinna nobilis*, successfully managing the bottleneck caused by limited sampling material due to the species' "Critically Endangered" status. The fast-CTAB DNA extraction method we propose, modified from [26], provides a streamlined and efficient approach for obtaining high-quality DNA suitable for amplifying long DNA fragments across different shell samples. This protocol coupled with the nested PCR approach using PnCOIMK22 specific primers results in excellent quality results for *COI* amplification.

The primary objective of this work was to develop and optimize molecular protocols specifically tailored for the accurate identification of the endangered mollusc *Pinna nobilis.* Unlike traditional bulk tissue sampling procedures commonly employed in DNA-based genetic studies, our protocol requires only a minute shell fragment. The nature (shell fragment instead of mantle) and the reduction in material usage are especially critical for conservation efforts and genetic monitoring of *Pinna nobilis*, a species of ecological and conservation importance that is facing significant population declines. This methodological

advancement is crucial, as precise genetic identification is foundational for conservation and biodiversity assessments. Effective DNA extraction and amplification methods facilitate the reliable identification and monitoring of individual organisms, even from minimal, non-invasively collected samples, thereby minimizing harm to already threatened populations. Such optimized protocols could have wide-ranging applications, including assessing population genetic health, determining species distribution and habitat usage, and enabling forensic analyses aimed at combating illegal trade and exploitation. Ultimately, the refinement of these molecular techniques significantly contributes to informed conservation strategies and enhances efforts to protect and restore populations of critically endangered marine species such as *Pinna nobilis*.

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Abbreviations

The following abbreviations are used in this manuscript:

PCR	polymerase chain reaction
COI	cytochrome oxidase subunit I

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