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Chief Editor José Paula

Coral reefs of Mauritius in a changing global climate

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Aims and scope: The *Western Indian Ocean Journal of Marine Science* provides an avenue for the wide dissemination of high quality research generated in the Western Indian Ocean (WIO) region, in particular on the sustainable use of coastal and marine resources. This is central to the goal of supporting and promoting sustainable coastal development in the region, as well as contributing to the global base of marine science. The journal publishes original research articles dealing with all aspects of marine science and coastal management. Topics include, but are not limited to: theoretical studies, oceanography, marine biology and ecology, fisheries, recovery and restoration processes, legal and institutional frameworks, and interactions/relationships between humans and the coastal and marine environment. In addition, *Western Indian Ocean Journal of Marine Science* features state-of-the-art review articles and short communications. The journal will, from time to time, consist of special issues on major events or important thematic issues. Submitted articles are subjected to standard peer-review prior to publication.

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Word from the Editor

The last couple of years have been a time of change for the Western Indian Ocean Journal of Marine Science. The journal has a new and more modern layout, published online only, and the editorial Board was increased to include more disciplines pertaining to marine sciences. While important challenges still lie ahead, we are steadily advancing our standard to increase visibility and dissemination throughout the global scientific community. The central objective of the journal continues focused on the Western Indian Ocean region and serving its growing scientific community.

We are pleased to start the publication of special issues of the journal, launched here with the publication of manuscripts from the University of Mauritius Research Week 2016. The special issues aim to contribute for advancing marine science in the WIO by focusing on specific themes, geographical areas or assembling contributions from scientific meetings. The editorial processes are exactly the same as for regular issues, with double peer-review, and guest editors are considered.

José Paula
Chief Editor

Editorial Note · Coral reefs of Mauritius in a changing global climate

The University of Mauritius Research Week (UoM RW) has been held on an annual basis since 2007 and was organized for the 9th time from 19-23 September 2016. The Research Week is geared towards dissemination of knowledge generated through research activities at the University and by relevant stakeholders in accordance with the UoM's vision of "*Excellence in Research and Innovation*". In line with national priorities, the UoM organizes this event to provide insightful research outcomes not only for the advancement of academic knowledge, but for the benefit of the community at large, through robust policy recommendations.

Out of the multiple submissions made during the UoM RW 2016, a number of manuscripts in the field of ocean/marine sciences were selected to be published in the Western Indian Ocean Journal of Marine Science (WIOJMS), as a special issue entitled "Coral reefs of Mauritius in a changing global climate". This issue is presented in the context of Mauritius being surrounded by a beautiful but delicate coral reef ecosystem, which provides ample ecosystem services contributing to the national economy, but which is subjected to extreme climatic events. Hence, in this special issue several contributions advancing our scientific understanding for sustainable use and management of marine resources in a globally changing marine environment are articulated. The original article by Mattan-Moorgawa *et al.* investigates the photo-physiology of diseased and non-diseased corals. Coral diseases are becoming more common on reefs worldwide due to both local and global stressors. Ramah *et al.* then present a short communication related to substrate affinity by two giant clam species found on the Mauritian coral reefs. Giant clams are under threat worldwide and information on their substrate affinity and habitat aims at providing insightful information towards their sustainable management. In addition, Nandoo *et al.*, in an effort to optimize nucleic acid extraction protocols from marine gastropods, present an original article based on a comparative study using the gastropod genera *Planaxis*, *Cypraea* and *Drupella*. These marine gastropods are ecologically important for coral reefs, especially the coral-eating *Drupella*. Moreover, given the importance of intertidal molluscs, Kaullysing *et al.* document the density and diversity of the benthic molluscs while comparing sheltered and exposed coastal habitats. Appadoo & Beeltah report on the biology of *Platorchestia* sp. (Crustacea, Amphipoda) at Poste La Fayette, Mauritius. Studies on Amphipod diversity and distribution are important especially since studies on marine biodiversity are scarce around Mauritius. Another original article by Ragoonaden *et al*. analyses the recent acceleration of sea level rise in Mauritius and Rodrigues. Such studies are more important than ever in the light of a globally changing marine environment with small island states faced with issues related to rising sea level. Two field notes, based on field observations, are presented by Bhagooli *et al.*, documenting a variety of coral diseases, and *Stylophora pistillata*-like morphotypes occurring around Mauritius Island, respectively. Kaullysing *et al.* also present a field note on coral-eating gastropods observed around Mauritius.

Apart from the local contributors, international collaborators also contribute two original articles in this special issue. Casareto *et al.* characterize the chemical and biological aspects of a coral reef of Mauritius focusing on benthic carbon and nitrogen fixation. These studies related to benthic productivity are important for understanding sustainability of coral reefs and/or lagoonal fisheries. On the other hand, Tokumoto *et al.* document the first detection of membrane progestin receptor (mPR)-interacting compounds from Mauritian coral reef and lagoonal seawater. They used cutting-edge technology to detect key regulators of reproduction in seawater. These contributions in terms of original articles, short communications, and field notes generate new scientific knowledge that may better inform policy and decision making in the field of coral reef studies and management in Mauritius, while contributing to the understanding of coral reefs in the wider Western Indian Ocean region.

Prof. Sanjeev K. Sobhee
Pro-Vice Chancellor (Academia)
The University of Mauritius

A comparative analysis of nucleic acid extraction methods on marine gastropods belonging to the genera *Planaxis*, *Cypraea* and *Drupella* from Mauritius

Aminah B. Z. Nandoo[1], Nawsheen Taleb-Hossenkhan[1]*, Ranjeet Bhagooli[2]

[1] Department of Biosciences, Faculty of Science, University of Mauritius, Réduit 80837, Republic of Mauritius

[2] Department of Marine & Ocean Science, Fisheries & Mariculture, Faculty of Ocean Studies, University of Mauritius, Réduit 80837, Republic of Mauritius

* Corresponding author: n.taleb@uom.ac.mu

Abstract

In recent years, molluscan systematics has increasingly relied on genetic tools to improve both traditional identification and classification methods. An essential prerequisite for molecular methods is obtaining sufficient quantities of high molecular-weight, relatively uncontaminated and minimally degraded DNA. Several nucleic acid extraction protocols have been reported for molluscan DNA extraction such as the phenol-chloroform and salting-out methods, but their relative efficiencies seem to vary between different genera of molluscs and no single protocol can be universally applied across all genera. We investigated the efficiencies of four nucleic acid extraction protocols (Methods I to IV) on the ecologically important marine gastropods belonging to the genera *Planaxis*, *Cypraea* and *Drupella* collected from Mauritius. Methods I - III are phenol-chloroform based while Method IV is based on binding to a selective silica membrane. We compared the efficiency in terms of purity, molecular weight and yield of nucleic acids extracted to investigate which protocol works best for which genus. We found that for each method, the nucleic acid yield varied significantly across the different genera, with Method I working best for *Cypraea* and *Drupella* and Method III working best on *Planaxis*. Based on these findings, we propose ways to optimise these two protocols.

Keywords: Nucleic acid extraction, *Planaxis, Cypraea, Drupella,* Mauritius

Introduction

The inventory of marine invertebrate macrofauna in Mauritian waters is still incomplete (Ministry of Agro-industry and Food Security, 2015) and little is known about the status of marine malacofauna in Mauritius. In the face of various anthropogenic activities such as destructive fishing techniques and rapid coastal development (Ministry of Environment and Sustainable Development, 2010) to which the vast Mauritian ocean territory is exposed, there is a great concern that most species will go extinct before they are actually described. Therefore, there is a pressing need to create baseline data about the diversity and distribution of marine molluscs in Mauritius and to monitor their conservation status, all of which require proper taxonomical identification and classification (Mace, 2004).

Corallivorous gastropods such as those belonging to the genus *Drupella* (Subclass: Hypsogastropoda) are found to cause significant damage to corals (Pillay *et al.*, 2012; Morton *et al.*, 2002; Hoeksema *et al.*, 2013) by removing calcium carbonate constituting their skeleton (Cumming & McCorry, 1998) and acting as vectors of brown band disease (Nicolet *et al.*, 2013). Hence, it is worthwhile to accurately identify *Drupella* gastropods at species level to determine the extent of coral damage caused by each species. Marine molluscs can also act as bio-monitors of pollution, and these organisms require specific identification (Rainbow, 1995). For instance, species belonging to the genus *Planaxis* (Subclass: Caenogastropoda), are reported to be efficient bio-indicators of pollution in coastal waters as they accumulate heavy metals

in their tissues and shell in proportion to those pollutants in their environment (Manavi, 2013). Gastropods belonging to the genus *Cypraea* (Subclass: Hypsogastropoda), have long been useful by humans as a form of money (Sundström & Hopkins, 1974) and have been heavily traded (Wood & Wells, 1995) and over-collected for their beautiful shells (Kay, 1995). With regards to the genera *Planaxis* and *Cypraea*, there are also doubtful phylogenetic claims (that have not been ratified thus far) that question the endemicity of some of the rare species found in Mauritius (Michel, 1988; Houbrick, 1987).

Gastropods can be identified using taxonomic morphological features like shell shape, radula, columellar teeth, reproductive structures and specific life stages (Caldeira *et al.*, 1998; Ponder & Lindberg, 1997; Barco *et al.*, 2010). However, relying on morphology alone to identify mollusc species can be complicated and, consequently, unreliable (Caldeira *et al.*, 2004; Packer *et al.*, 2009). For example, the frequent abrasion by sand might result in loss of teeth (Michel, 1988). Moreover, the phenomenon of phenotypic plasticity and the presence of cryptic species (Hebert *et al.*, 2003) further complicate morphological identification of gastropod species.

Molecular methods provide a modern and more accurate approach to molluscan systematics. They are helpful in solving phylogenetic relationships and understanding evolution of metazoans among different taxa and phyla (Garey & Schmidt-Rhaesa, 1998). The first step of any genetic study is the extraction of sufficient amounts of high molecular weight, relatively uncontaminated and minimally degraded DNA. Mollusc tissues are known to be difficult to work with because of their size and limited amount of 'suitable' tissues for DNA extraction, the physiology of tissue used for DNA extraction (Pereira *et al.*, 2011), and the presence of large amounts of mucopolysaccharides and polyphenolic proteins that co-purify with DNA and negatively impact downstream applications (Winnepenninckx *et al.*, 1993). Hence, standard extraction protocols have to be extensively modified and optimized to adapt to molluscan tissue, and reports of such protocols are scarce (Pereira *et al.*, 2011). Moreover, it appears that the efficiency of the few available protocols vary across genera as differences in quantity and quality of nucleic acids are reported when the same methods are used on different genera. Results might vary further if the species originate from marine environments.

In this study, we performed a comparative analysis of four nucleic acid extraction protocols, named Methods I, II, III and IV, to determine which protocol was best suited for each of three genera, and how the various protocols could be combined and/or modified to achieve better efficiency. These methods have been formally reported to work on molluscs and other marine organisms, and on marine gastropods belonging to the genera *Planaxis*, *Cypraea* and *Drupella* collected from Mauritian lagoons.

Materials and methods

The gastropods were identified based on reported morphological features (Fig. 1) from different lagoons in Mauritius, handpicked, and their lengths measured using a digital Vernier caliper (Table 1). The individuals were transported in ice from field to laboratory where they were kept at -20°C until nucleic acid extraction was carried out within 24 hours. The shell of each individual gastropod was cracked using sterile pliers to reveal the foot muscle tissue (Fig. 2) which was removed and weighed. The protocols described

Table 1. Gastropods identified and handpicked from different substrates in different lagoons of Mauritius, with their sizes measured and averaged.

Genus	Morphological features described by	Substratum	Lagoon	Collection Date	Number of individuals collected	Average size of individuals (±0.02 mm)
Planaxis	Houbrick (1987) pp. 5-6	Rock surfaces and crevices	Pointe-aux-Piments	November 2015	23	24.66
Cypraea	Michel (1988) pp. 42	Sediment	Palmar	November 2015	46	18.03
Drupella	Johnson and Cumming (1995)	*Porites* coral	Palmar	November 2015	3	21.18
		Acropora coral	Flic-en-Flac	February 2016	6	

Table 2. Comparison of the main steps involved when each nucleic acid extraction protocol was carried out. Each protocol involved six major steps: breaking and processing mollusc tissues; lysis of tissues; nucleic acid extraction; precipitation; washing; and elution. The table illustrates the specific reagents, the volumes used and the incubation periods for each sub-step of the extraction process. "Yes" indicates that the sub-step was carried out whereas "No" indicates that the sub-step was not carried out. "PK" stands for Proteinase K. The total number of hours to complete a single protocol with triplicate samples is indicated at the bottom of the table.

Main steps	Sub-step(s)	Method I – Sokolov (2000) protocol	Method II – Zamoum & Furla (2012) protocol	Method III – Geist et al (2008) protocol	Method IV – QIAGEN® DNeasy Blood and Tissue Kit
Step 1: Treatment of tissues before lysis	Homogenisation of tissue using a mini-tissue homogeniser	Yes	No	No	No
	Cutting tissue into small pieces using scissors	No	No	No	Yes
	Addition of 500µl NaOH	No	Yes	No	No
Step 2: Lysis	Composition of lysis buffer in 1MTris-HCl pH8	1%SDS 0.1MNaCl 0.01M EDTA 2000-4000 µg PK	6%SDS 0.4MNaCl 0.02 M EDTA 40µg PK	0.5%SDS 0.1MNaCl 0.1M EDTA 250µg PK	180 µl ATL buffer 400µg PK 200 µl buffer AL
	Incubation time (hours)/ temperature (°C) in lysis buffer	2 hours at 55°C	2 hours at 56°C	12 hours at 56°C	2 hours at 56°C
	Addition of 100µl saturated KCl after incubation period	Yes	No	No	No
Step 3: Nucleic acid extraction	Number of phenol/ chloroform/ isoamyl alcohol (25:24:1) extractions	2	1	1	0
	Number of chloroform-only extractions	0	0	1	0
Step 4: Precipitation	Reagents, temperature and time	Ice-cold isopropanol at 4°C overnight	Ice-cold isopropanol at -20°C overnight	Ice-cold isopropanol at 4°C overnight	200µl 90% ethanol
Step 5: Washing	Reagents	500 µl 70% ethanol	500 µl 70% ethanol	500 µl 70% ethanol	500µl buffer AW1, 500µl buffer AW2
Step 6: Elution	Buffer used for storage	100 µl TE buffer	50 µl TE buffer	100 µl TE buffer	200 µl AE buffer
Total time (hours)		~ 28	~ 29	~ 37	~2.5

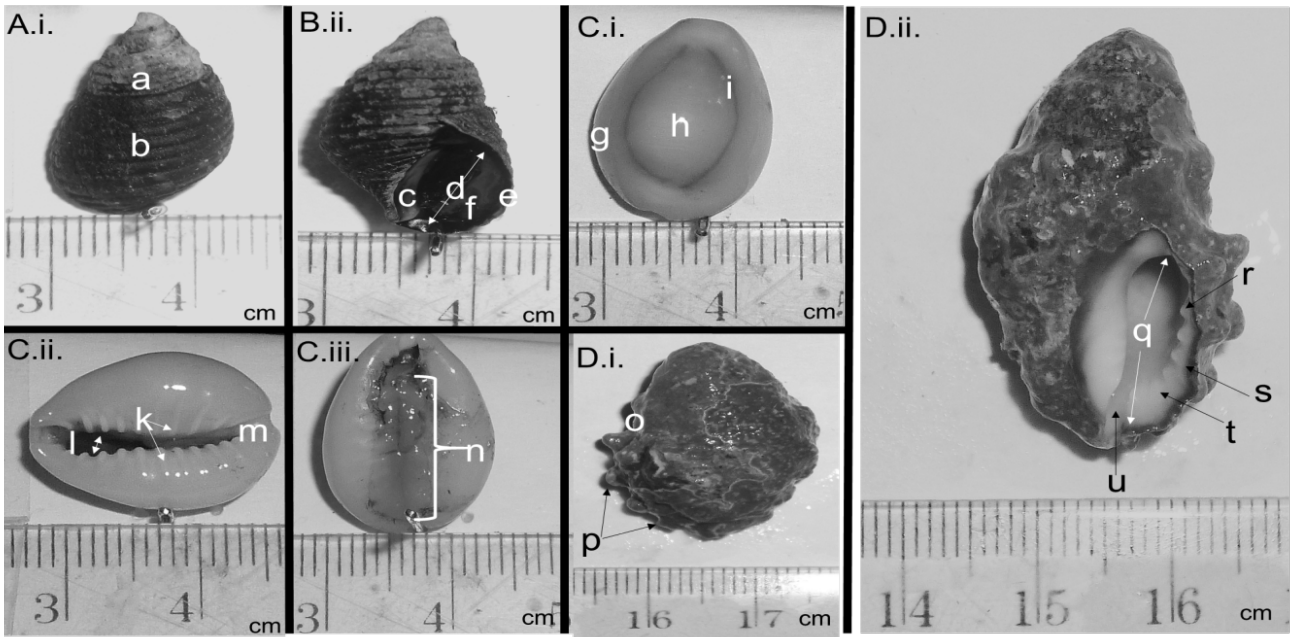

Figure 1. Morphology of one individual representing each genus. A.i.: Dorsal view of *Planaxis sulcatus* gastropod (a=Walls with incised spiral lines and grooves, b=large and wide body wall); B.ii. Ventral view of *P.sulcatus* (c=Whitish-purple columella, d=ovate aperture, e=smooth denticulate outer lip, f=purple inner lip); C.i.: Dorsal view of *Cypraea annulus* (g=Round lateral margins, h=smooth and polished surface, i=yellow dorsal ring); C.ii. Ventral side of *C. annulus* without foot muscle tissue (k=columellar teeth, l=aperture, m=anterior canal); C.iii. Ventral side of *C. annulus* showing n=foot muscle tissue); D.i. Dorsal side of *Drupella* (o=deeply incised suture, p=spines); D.ii. Ventral side of *Drupella* (q=aperture, r= radular tooth, s=thin outer lip, t= thick outer lip, u=siphonal canal).

by Sokolov (2000) (Method I); Zamoum & Furla (2012) (Method II); Geist *et al*. (2008) (Method III); and the commercial, QIAGEN® DNeasy Blood and Tissue kit (Method IV), were conducted in triplicates from individuals belonging to each genus studied. However, the protocols were slightly modified according to laboratory equipment and time available, as described in Table 2.

The efficiency of four different nucleic-acid extraction methods (Methods I-IV) on three genera of marine gastropods (*Planaxis, Cypraea* and *Drupella*) was evaluated. Method I entailed a two-step phenol-chloroform-based protocol with homogenization of the tissue, a 2-hour lysis incubation period, and addition of saturated KCl after the lysis. Method II entailed a 1-step phenol-chloroform-based protocol with a 2-hour alkaline lysis step without tissue homogenization. Method III entailed a 1-step phenol followed by a 1-step chloroform-based protocol with an overnight lysis step without tissue homogenization. Method IV entailed a column-based method with a 2-hour lysis step without tissue homogenization.

The yield, concentration and purity of nucleic acids extracted were determined using measurements by UV spectrophotometry at 260 nm and 280 nm. Nucleic acids were checked for integrity by loading

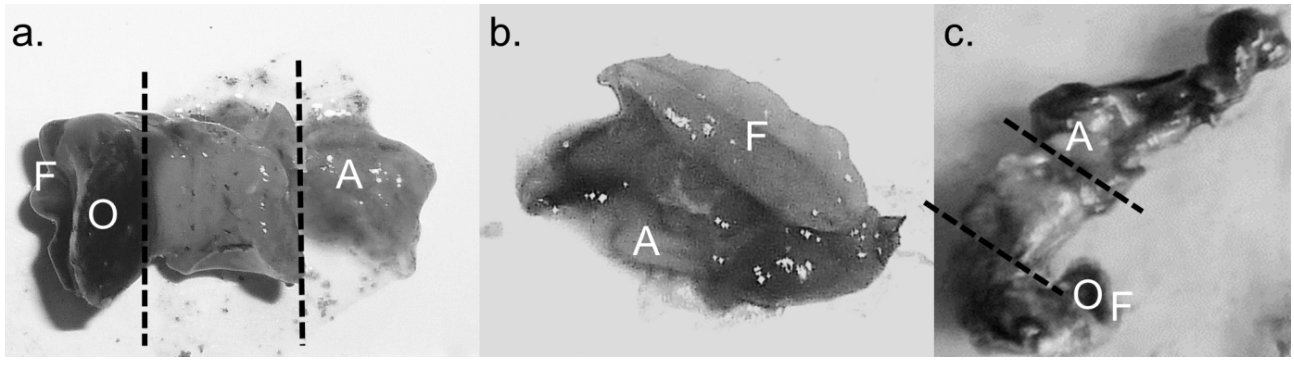

Figure 2. Part of gastropod tissue used for nucleic acid extraction a. *Planaxis*; b. *Cypraea*; c. *Drupella* (F = Foot muscle tissue, O = Operculum, A = Alimentary tract). Recommended region to be used for nucleic acid extraction is shown between the two dotted lines.

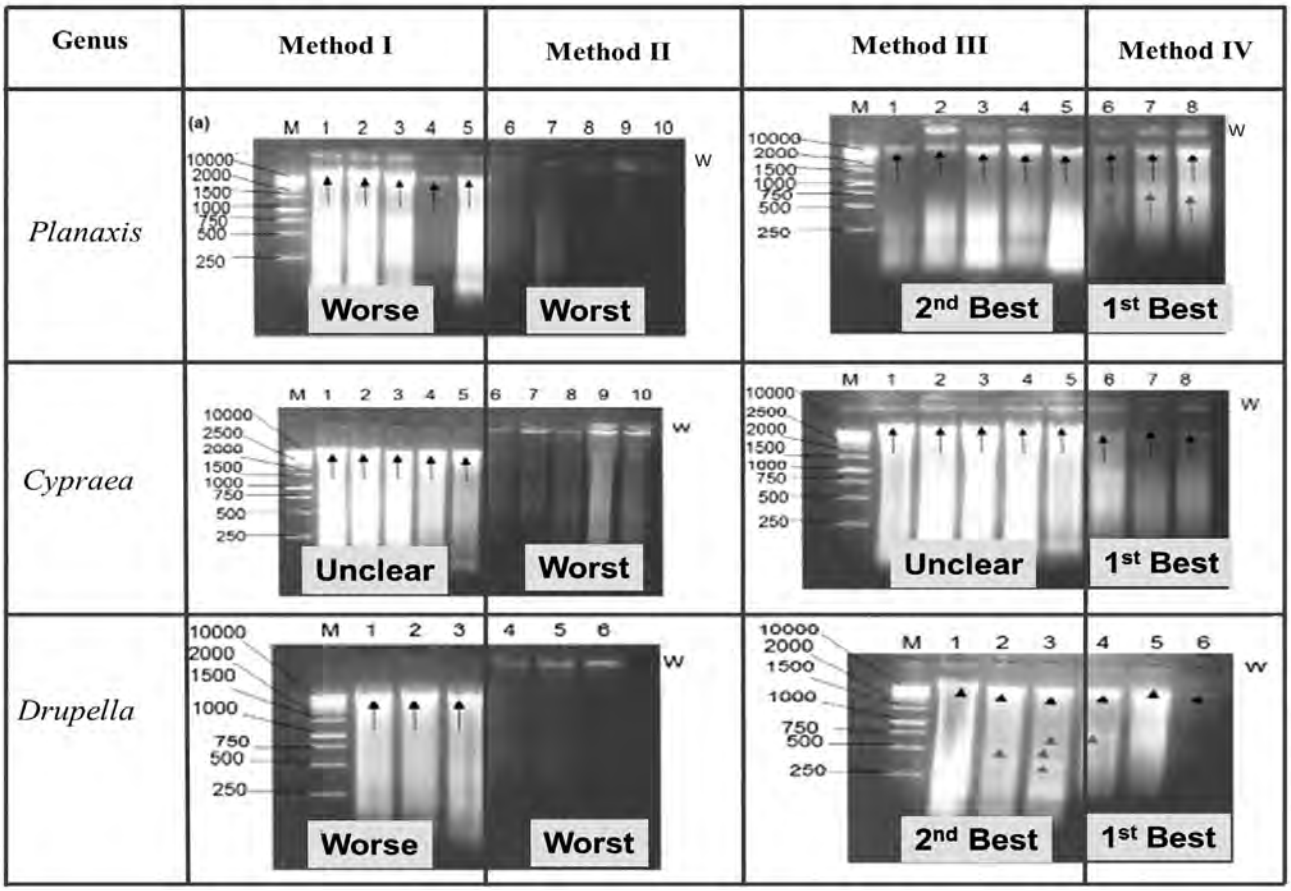

Figure 3. Assessing nucleic acid integrity by 1.5% Agarose gel electrophoresis (M=Gene Ruler 1Kb DNA Ladder, W=Position of wells).

into a 1.5% agarose gel stained with ethidium bromide, electrophoresed, and viewed under ultraviolet light.

Results

Nucleic acid integrity

Figure 3 shows the results of agarose gel electrophoresis of all four different methods for the three selected marine gastropods studied. It was observed that Methods I, III, II successfully extracted high molecular weight nucleic acids of 10,000 Kbp in all marine molluscs investigated, as indicated by the black arrows. However, the nucleic acids extracted using Methods I and III were degraded, as depicted by the bright white smears. Method IV extracts least degraded nucleic acids, hence described as the "1st Best" method in all genera, for nucleic acid integrity. Among the phenol-chloroform methods, it can be observed that the degree of degradation is different for different genera of marine molluscs, indicating that the efficiency of the protocols is dependent on the nature of the tissue used. For example, for *Planaxis* Method III extracts less degraded nucleic acids as compared to Method I. For *Cypraea*, Methods I and III extract nucleic acids degraded to the same extent, hence described "unclear". For *Drupella* it can be observed that Method III extracts nucleic acids of comparatively less degraded quality than Method I. Additionally, it extracts some other nucleic acid fragments of smaller than 10000 Kbp as indicated by the arrowheads. Finally, Method II displays negative results in all genera, by showing no clear and distinct bands of nucleic acids, clearly showing that this is not a method of choice for extraction of nucleic acids from marine gastropods.

Interaction between parameters

A two-way ANOVA was carried out to determine whether the percentage nucleic acid yield and/or nucleic acid purity are statistically different when the four protocols were tested on each genus (Table 3). The biochemical composition and physiology of the tissues from the different genera affects nucleic acid purity. On the other hand, the *modus operandi* of the protocol affected the percentage nucleic acid yield. The overall interaction between the genus and protocol affected both parameters. Table 4 displays the statistical tests performed (One way ANOVA or Kruskal-Wallis H-test) to determine whether the percentage nucleic acid yield and/or purity are statistically different when different

Table 3. *p*-values of Two-way ANOVA carried out to determine whether percentage nucleic acid yield and nucleic acid purity depend on genus only, protocol only, or an interaction between both (α = 0.05) (*= statistically significant results).

	Parameters to assess efficiency of protocols	
	Percentage nucleic acid yield	Nucleic acid Purity
Genus	0.336	0.030 *
Protocol	0.005*	0.095
Genus-protocol interaction	0.010 *	0.022*

Table 4. *p*-values or asymptotic values obtained from One way ANOVA and Kruskal Wallis H-test respectively, to determine which parameter should be used to determine which protocol works best for each genus (α = 0.05) (*=P<0.05; **=P<0.01; ***=P<0.001).

Genus	Parameter	*p*-value (ANOVA)/Asymptotic value (Kruskal Wallis H-test)
Cypraea	%Nucleic acid yield	0.007**
	Nucleic acid purity	0.081
Planaxis	% Nucleic acid yield	0.000***
	Nucleic acid purity	0.214
Drupella	% Nucleic acid yield	0.013*
	Nucleic acid purity	0.088

protocols are used on each genus, and which parameter can be used to determine which protocol works best for each genus. In all cases, the best protocol for each genus is based on percentage nucleic acid yield.

Nucleic acid purity

Table 5 illustrates the nucleic acid purity values derived from calculating the ratio of absorbance at 260 nm over absorbance at 280 nm. Ideal DNA purity values normally lie in the range 1.8-2.0. Methods I and III extract relatively pure nucleic acids in all the genera investigated. However for Method IV, the purity of nucleic acids is contaminated with values much greater than 2.0 for *Planaxis* and *Cypraea*, and negative values for *Drupella*. Method IV was therefore removed from remaining analyses.

Nucleic acid yield

Nucleic acid quantity is determined by nucleic acid yield, expressed by the percentage nucleic acid yield which is the amount of nucleic acids that can be extracted from a known mass of starting tissue. The percentage nucleic acid yield, therefore, indicates how much starting tissue is required by the protocol.

Figure 4 displays the percentage nucleic acid yields obtained from each protocol for each genus. For *Planaxis*, the highest-yielding protocol is Method III. For *Cypraea* the highest-yielding protocols are Method I, Method III and Method IV, in descending order of percentage yields. Finally, for *Drupella*, Method I is the best protocol.

Table 5. Nucleic acid purity.

	Nucleic acid purity (Abs_{260}/Abs_{280})			
	Method I	Method II	Method III	Method IV
Planaxis	1.90±0.91	0.40±5.19	2.30±0.85	2.50±1.69
Cypraea	0.70±2.50	0.80±2.54	1.80±0.13	2.10±0.51
Drupella	2.10±0.43	0.90±0.51	1.90±0.44	0.30±2.61

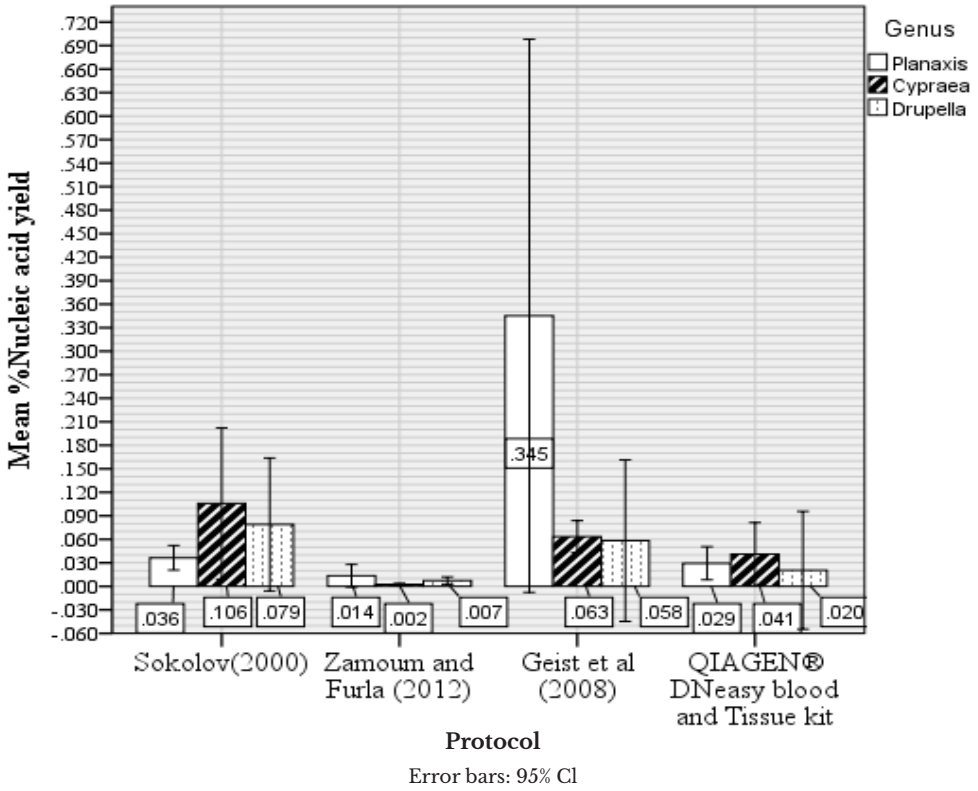

Figure 4. Percentage yields of nucleic acid extracted.

Discussion

From Table 3 it can be seen that that the nature and physiology of the gastropod tissues affect nucleic acid purity, whereas the *modus operandi* of each protocol affects the percentage nucleic acid yielded. Together, the interaction between the protocol and the nature of tissues significantly affect the percentage yield and the purity of nucleic acids. From Table 4 it is apparent that different percentages of nucleic acid yield were obtained when different protocols are tested on the same genus. No differences were obtained between values of nucleic acid purity when these protocols are tested in each genus. Therefore percentage nucleic acid yield will be used as a parameter to demarcate which protocol works best within each genus of gastropod. In terms of percentage nucleic acid yield (Fig. 4) and integrity (Fig. 3), Methods I and III worked best compared with the other protocols tested. Based on percentage nucleic acid yield, Method III clearly yielded the best results for *Planaxis*, while Method I was most suited for *Cypraea* and *Drupella*. The main difference in Method III is the overnight lysis step, compared to a 2 hour lysis period for all the other methods (Table 2). Although longer incubation period makes a considerable difference in the nucleic acid yield (Miller *et al*, 1999) for *Planaxis*, it does not make a significant difference for yield in the other genera. This implies that Method III, compared with the other protocols, has the correct steps to remove the unwanted chemicals from tissues of *Planaxis*. Therefore, when working with the genus *Planaxis*, an overnight incubation lysis step should be used, while a 2-hour lysis step can be maintained for other genera including *Cypraea* and *Drupella*. Conversely, in terms of percentage nucleic acid yield, Method I works best for *Cypraea* and *Drupella* which means that the protocol is, comparatively, better adapted to work on these mollusc tissues. This could be because of the additional high-salt potassium chloride (KCl) treatment after lysis to further remove most polyphenols and polysaccharides that may be present (Porebski, 1997). Proteinase K (PK) is a powerful enzyme that can be used to instantaneously lyse cellular membranes of eukaryotes (Blin & Stafford, 1976). The fact that Method I, which includes the highest concentration of PK in the lysis buffer compared to all other methods, yielded the best yield of nucleic acid for *Cypraea* and *Drupella* might indicate that tissues of marine gastropods belonging to these genera contain a higher amount of protein and would therefore benefit from a higher amount of PK added to the lysis buffer of choice. Moreover, it has been reported that PK is very active in the presence of the detergent Sodium Dodecyl Sulphate (SDS) (Gross-Bellard *et al*, 1973), and the lysis buffer of Method I contains the

highest amount of SDS compared with Method III, which activates the enzyme to promptly lyse the layers of tissue in comparatively less time than Method III. In this regard, at least 1%SDS should be used in a lysis buffer for work on marine molluscs. Nucleic acid yield can be an issue when the amount of 'usable' tissue from the selected marine gastropod is limited, especially in cases where the species is rare or facing population decline, or is claimed to be endemic and/or protected (such as *Planaxis piliger* and *Cypraea mauritiana* that are claimed to be endemic to the Mascarene Islands and Mauritius respectively (Houbrick, 1987; Michel, 1988). In these cases, methods that achieve high percentage yields of extracted nucleic acids should be favoured so that a small amount of tissue would be sufficient to extract a sufficient amount of nucleic acid for downstream applications.

The quality (or integrity) and size of nucleic acid extracted was estimated based on agarose gel electrophoresis. The extent of degradation of nucleic acids can be determined by the appearance of smears (Michaelis *et al*, 2008) and the sizes of the bands indicate the molecular weight of the nucleic acids extracted (Yun Lee *et al*, 2012). It is expected that highly degraded DNA will be unsuitable for downstream applications such as Polymerase Chain Reaction (PCR), whereby primers cannot anneal properly if the template DNA is made up of small fragments (Golenberg *et al*, 1996). Similarly, highly degraded DNA cannot be used for Restriction Fragment Length Polymorphism (RFLP) reactions, because restriction sites can be lost if the DNA is sheared into small fragments of 2-4 kbp in length (Rudin & Inman, 2002). In this study, it was found that Method III and Method IV were the two best protocols to preserve nucleic acid integrity (Fig. 3) by allowing the extraction of high-molecular weight nucleic acids across all three genera of marine gastropods. Both of those methods did not involve homogenization of the starting tissue. Therefore, although Method I is best for yield in *Cypraea* and *Drupella*, the integrity of the extracted nucleic acids is compromised, most probably because of the tissue homogenization step. Methods III and IV simply involve cutting the tissue into small pieces and allowing it to lyse gently, without physical shearing, which yields nucleic acids of higher integrity. Another factor that could affect nucleic acid integrity is that all mollusc samples collected in the field were frozen at -20°C and then defrosted when needed to be used for nucleic acid extraction. It has been reported that repeated freeze-thaw cycles damage nucleic acid quality (Lahiri & Schnabel, 1993). Hence it is recommend that freshly harvested samples are used to extract nucleic acids. Addition of Ethylene-Diamine-Tetra-Acetic acid (EDTA) to the lysis buffer minimises degradation (Lahiri & Schnabel, 1993). Therefore, the 0.01-0.1M EDTA is imperative in any lysis buffer to be used for mollusc tissues. Alternatively, for long-term storage, marine molluscs can be preserved in 95% ethanol until nucleic acid extraction, a preservation method that has recently been shown not to inhibit downstream applications, including DNA barcoding (Stein *et al*., 2013)such as DNA barcoding, have the potential to enhance biomonitoring programs worldwide. Altering routinely used sample preservation methods to protect DNA from degradation may pose a potential impediment to application of DNA barcoding and metagenomics for biomonitoring using benthic macroinvertebrates. Using higher volumes or concentrations of ethanol, requirements for shorter holding times, or the need to include additional filtering may increase cost and logistical constraints to existing biomonitoring programs. To address this issue we evaluated the efficacy of various ethanol-based sample preservation methods at maintaining DNA integrity. We evaluated a series of methods that were minimally modified from typical field protocols in order to identify an approach that can be readily incorporated into existing monitoring programs. Benthic macroinvertebrates were collected from a minimally disturbed stream in southern California, USA and subjected to one of six preservation treatments. Ten individuals from five taxa were selected from each treatment and processed to produce DNA barcodes from the mitochondrial gene cytochrome c oxidase I (COI. Besides, it is possible that the foot muscle tissue used contains a large amount of dead cells before nucleic acid extraction, probably due to crawling with limited lubricant secreted by the mollusc. It is therefore recommended that the region between the foot muscle tissue and the alimentary tract is removed (Fig. 2) for nucleic acid extraction in marine gastropods instead of the foot muscle tissue.

The third aspect considered in this study was nucleic acid purity, which indicates the level of contamination present in the extracted sample. Impure samples constrain downstream applications, like PCR, by acting as inhibitors (Besseti, 2007). Besides, there seems to be a positive correlation between the number of organic-solvent extraction steps and the purity of the extracted nucleic acids, as including more organic-solvent steps will remove more impurities from the lysis

extract. Methods I and III involve two such organic-solvent extraction steps, and Method II involves just one. Since there is no statistically significant differences for mean purity values between the various protocols tested, the number of organic-solvent extracting steps can be kept to one when working with the three genera of marine gastropods tested. Since each phenol-based extraction step causes the loss of some the soluble nucleic acids (Liu, 2009), minimising the number of organic-solvent extraction steps not only minimises damage to DNA (Mater methods, 2013) but also will increase the yield of DNA extracted.

Based on the results of this study, it is recommended that a consolidated protocol is used which should yield good results for DNA yield, purity and integrity for most species of marine gastropods. This consolidated protocol is essentially based on the one published by Sokolov (2000) with some modifications. Firstly, the gastropod tissue should not be homogenized mechanically, but simply cut into small pieces and left in the lysis buffer for gentle lysis. Secondly, lysis should be performed overnight at a temperature of *55-56*°C (also recommended by Huelsken *et al*., 2011). Thirdly, the concentration of PK should be at least 0.04 mg/ml in the lysis buffer containing at least 1% SDS. Finally, only one phenol/chloroform/isoamyl alcohol extraction step can be performed followed by a chloroform-only step (also recommended by E.Z.N.A®). The use of an additional sodium hydroxide treatment is not recommended for tissues of marine gastropods. It is also recommended that nucleic acids extracted in aliquots are stored for further analyses so that repeated freeze-thaw cycles can be minimised. The results of this study provides useful information for developing new protocols for mucopolysaccharide-rich marine gastropods.

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