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COI amplification success from mucus-rich marine gastropods (Gastropoda: Naticidae) depends on DNA extraction method and preserving agent

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Abstract: In this study we describe a DNA extraction method that combines the advantages of the traditional CTAB extraction with those of DNA extraction kits by leaving out hazardous reagents such as SDS and phenol. We furthermore describe adequate conservation solutions usable in the field and in the lab for the efficient fixation of moon snail tissue to be used for DNA extraction and PCR amplification.

Keywords: CTAB, DNeasy Blood & Tissue Kit, tissue fixation, gastropoda, mucus

Zusammenfassung: Die DNA-Extraktion ist einer der wichtigsten Schritte in genetischen Anwendungen bei der Analyse der Taxonomie und Phylogenie. Dabei hat sich herausgestellt, dass die Polymerase-Kettenreaktion von aus Mollusken extrahierter DNA durch mitaufgereinigte Polysaccharide und akkumulierte Inhibitoren gehemmt oder völlig verhindert wird. In dieser Studie beschreiben wir die erfolgreiche Anwendung einer Kombination zweier vielfach bei Gastropoden verwendeter DNA-Extraktionsmethoden - dem DNeasy Blood & Tissue Kit (Qiagen) mit anschliessender CTAB-Aufreinigung der DNA. Für Mondschnecken (Naticidae) erreichen wir damit reproduzierbar hohe DNA-Ausbeuten und Amplifikationsraten. Des Weiteren präsentieren wir Ergebnisse einer Untersuchung von 9 verschiedenen Konservierungslösungen, die zur Fixierung von Gastropoden im Labor und im Feld eingesetzt werden können. Dabei stellte sich heraus, dass 100 % Ethanol p.A., RNAlater (Qiagen) und Sambuca am Besten dazu geeignet waren, um auch noch nach ca. 20 Wochen erfolgreich mitochondriale DNA aus Mondschnecken zu amplifizieren. Verdünnte Alkohole und Methanol ließen eine Amplifikation noch 25 Tage nach der Fixierung zu, während eine Amplifikation von in Isopropanol oder Formalin fixiertem Gewebe nur nach maximal 15 Tagen möglich war.

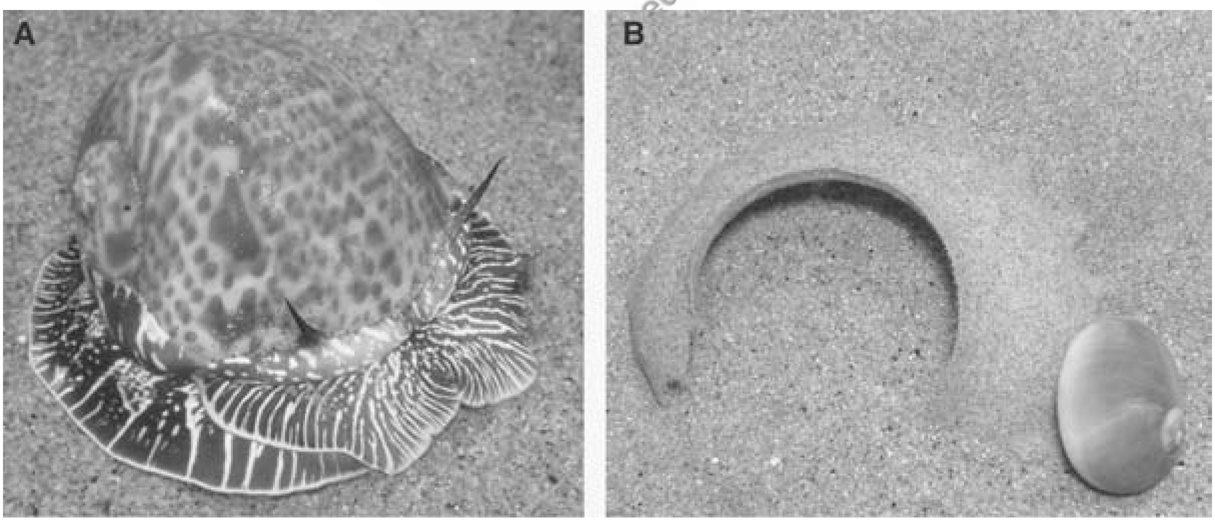

Fig. 1. Specimens analysed in this study. A. *Naticarius hebraeus* (Isola del Giglio, Italy; ca. 30 mm shell size). B. Egg mass and shell of *Neverita josephinia* (Isola del Giglio, Italy; diameter egg mass – ca. 30 mm).

Introduction

The use of molecular techniques has had a profound impact on systematic zoology and phylogenetic research. Many recent studies have shown that molecular phylogenetic analysis is a powerful tool to elucidate the evolution of the molluscan fauna (e.g., STEINER 1995; HARASEWYCH 1998; KOEHLER & al. 2004; COLGAN & al. 2006; WILLIAMS & OZAWA 2006; HUELSKEN & al. 2006, 2008). The discovery of cryptic species (e.g., COLLIN 2005; HEBERT & al. 2003a, 2003b), the identification of biogeographical distributions of species (e.g., RIGINOS & CUNNINGHAM 2005), the analysis of gene flow between populations (e.g., WILKE & DAVIS 2000), and the reconstruction of robust phylogenetic relationships (e.g., HARASEWYCH 1998; COLGAN & al. 2006) all benefit from a molecular approach. However, the utilization of genetic data for molecular systematics requires an efficient DNA isolation protocol and accurate and consistent PCR amplification to allow for maximal exploitation of freshly collected material as well as long-term stored material available in museum repositories. Efficient, reproducible protocols are also important to avoid species misidentification, erroneous taxa designation, and false species assignments in phylogenetic trees.

Apparently, DNA isolation is problematic in gastropods (SKUJIENÉ & SOROKA 2003; WILLIAMS 2007) and hampers the application of modern sequence analysis techniques in gastropods, thus impeding the study of their evolution. In general, two major technical issues affect the performance of molecular techniques: 1) tissue fixation and storage, and 2) DNA extraction (SCHANDER & HALANYCH 2003; MIETHING & al. 2006; NAGY 2010).

Although several methods have been published to extract DNA from formaldehyde-fixed tissues (see CHASE & al. 1998; SCHANDER & HALANYCH 2003), it is generally accepted that formaldehyde fixation degrades DNA and thus makes it difficult to isolate adequate amounts of high quality DNA of sufficient length (SCHANDER & HALANYCH 2003). But even if tissues have been fixed adequately in dehydrating agents (e.g., ethanol [EtOH]), the occurrence of mucopolysaccharides and uncharacterized PCR inhibitors in gastropod tissue pose difficulties in performing reliable, reproducible gene fragment amplification (SKUJIENÉ & SOROKA 2003; WILLIAMS 2007). This is particularly true for members of the species-rich gastropod family Naticidae (Caenogastropoda: Naticoidea) which are known to produce large amounts of mucus and take up considerable amounts of sea water, thus accumulating high concentrations of potentially inhibitory substances or toxic metabolites (e.g., tetrodotoxin) (HWANG & al. 1991). To overcome these unique challenges, several DNA extraction techniques have been designed to remove the mucus and inhibitors (e.g., DOYLE & DOYLE 1987; DOURIS & al. 1998; WINNEPENNINCKX & al. 1993; SOKOLOV 2000; SKUJIENÉ & SOROKA 2003).

However, for the Naticidae (Fig. 1A) these techniques often result in inadequate amounts of isolated DNA or in DNA that is not amenable to gene fragment amplification. Similar results have been obtained for naticid egg masses (Fig. 1B) which also consist of large amounts of mucus and therefore are difficult to analyse. The development of commercial DNA extraction kits has greatly simplified the extraction of total DNA from animal tissues and has successfully been applied to DNA extraction also from mucus-containing gastropods, providing adequate DNA for a number of molecular methods (see SKUJIENÉ & SOROKA 2003). Despite providing large amounts of DNA, the application of these extractions kits to naticid tissue, however, did not result in satisfactory levels of amplification success (Fig. 2C), thus presenting a problem for phylogenetic analyses and barcoding studies. Thus, for our studies of naticid phylogeny a more efficient extraction method was needed to obtain adequate amounts of high-quality DNA from these particularly mucus-rich animals. To achieve this, certain commonly used hazardous reagents such as SDS and phenol were avoided since they can inhibit the DNA polymerase during PCR (PEIST & al. 2001).

To obtain DNA of the required high quality, it is best extracted from fresh tissue. During field work, processing fresh tissue immediately for DNA isolation is not always possible, which is why adequate tissue fixation and storage is crucial for high quality DNA extraction from such samples. The storage of the tissue in different preservatives results in different amplification success rates that clearly depend on the preservative used as well as on storage duration (SCHANDER & HALANYCH 2003). To date, EtOH is the most commonly used preservative for tissue fixation in molecular systematics. EtOH dehydrates the tissue and minimizes nuclease activity that would degrade nucleic acids (TOWNSON & al. 1999). However, highly concentrated EtOH may not always be a viable option for field work as

EtOH is prohibited on airplanes and requires complicated and costly regulations when sent by mail. One option is to initially preserve the tissue in highly concentrated EtOH and replace it with diluted EtOH for shipment or transport. WILLIAMS (2007) demonstrated that a reduction from 100% EtOH to 24% EtOH for a duration of 3 to 15 days resulted in adequate recovery of total DNA and produced high yield amplification products of the nuclear 18S rRNA gene. Alternative preservation agents (e.g., DMSO) have successfully been employed during field work but may be unsuitable for museum or long-term storage as the long-term effects of storage in these solutions are unknown (WILLIAMS 2007).

COI is currently one of the most widely used genes for phylogeny, systematics, and the identification of species. For gastropod tissues, however, there is only little information available on the amplification success of the COI gene fragment as a function of preservation, storage media, storage duration, or DNA extraction (e.g., SKUJIENÉ & SOROKA 2003). We therefore demonstrate that reliable PCR amplification of COI gene fragments from mucus-rich naticid specimens is highly dependent on the applied extraction method, the preservative used, and the storage duration of the preserved tissue.

Material and methods

DNA extraction method

Five tissue samples were isolated from the foot of a 100% EtOH-fixed specimen of *Polinices mellosus* (HEDLEY 1924) from Lizard Island (Queensland, Australia) for each DNA extraction analysis (Figs. 2A-C). Field work was conducted in accordance with the Great Barrier Reef Marine Park Authority (GBRMPA) permit G05/16526.1 to THOMAS HUELSKEN and MICHAEL HOLLMANN. The DNA was extracted using 1) the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions, 2) the CTAB (Cetyl trimethylammonium bromide) extraction method (after DOYLE & DOYLE 1987), and 3) a combination of both methods.

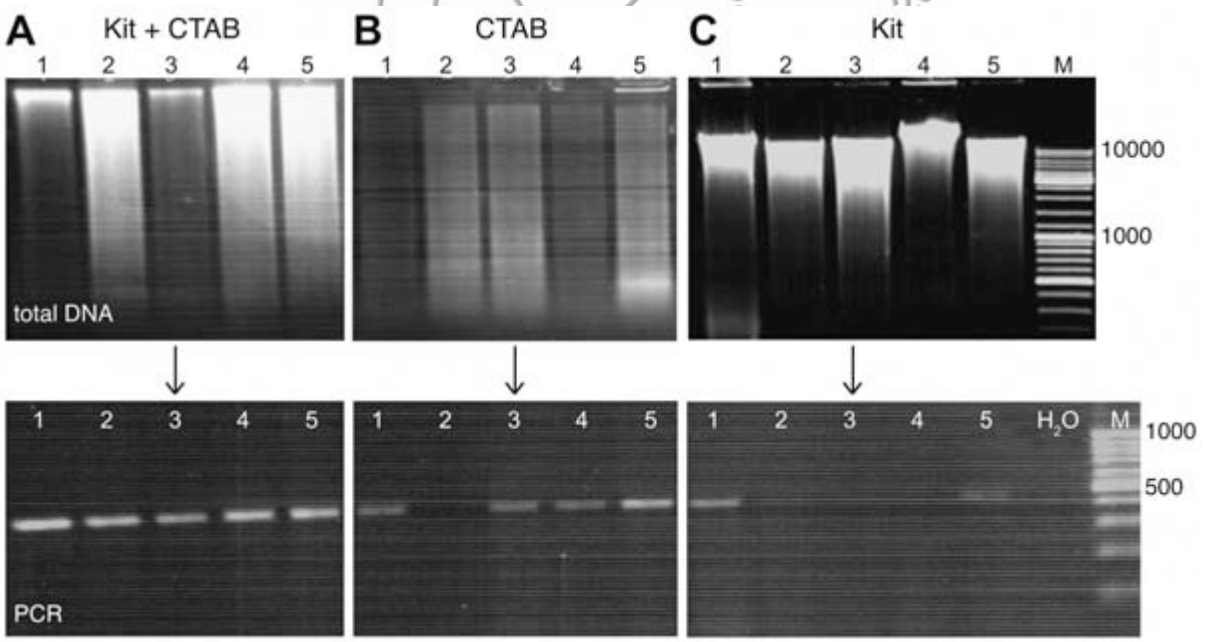

Fig. 2. Extracted DNA and resulting amplification success visualized on ethidium bromide-stained agarose gels. A. DNA obtained using the "combination method" for extraction (DNeasy Blood and Tissue Kit + CTAB purification). B. DNA extracted using only the CTAB extraction method. C. DNA extracted using only the DNeasy Blood and Tissue Kit (Qiagen). M. Molecular weight marker.

The "combination method" consists of a DNA extraction using the DNeasy Blood and Tissue Kit followed by a CTAB purification step. For this, the DNA solution obtained with the extraction kit was mixed thoroughly with one vol. of 2x CTAB buffer and incubated for 2-4 hours on a heating block at 60 °C. The sample was then purified via a chloroform-isoamyl alcohol precipitation (DOYLE & DOYLE 1987) by adding one vol. of chloroform-isoamyl alcohol (24:1). The mixture was shaken softly for

2 minutes and centrifuged at 16.000 xg for 10 minutes. The aqueous upper phase was transferred to new reaction tubes. The chloroform-isoamyl alcohol extraction was repeated once before the DNA was precipitated over night from the aqueous phase by adding 2.5 vol. ice-cold (-20 °C) 100% EtOH and 1/10 vol. 3 M sodium acetate (NaOAc) and centrifuged at 16.000 xg for 30 min. The supernatant was aspirated and the DNA pellet was re-dissolved in 50 µl of 0.1x TE and placed at 50 °C for 30 min to facilitate resuspension.

A 447 bp fragment of the COI gene was amplified from each DNA sample in a total reaction volume of 50 µl in a MJ Research thermocycler (Watertown, MA, USA) using *Taq*-Polymerase (Invitrogen, Karlsruhe, Germany). The following PCR program was used: an initial single cycle of 95 °C for 5 minutes, 39 °C for 45 seconds, and 72 °C for 1 minute; followed by 39 cycles of 95 °C for 20 seconds, 39 °C for 20 seconds, and 72 °C for 35 seconds. Amplification primers used were P388 (5´-GCTTTTGTTATAATTTTYTT-3´) and P390 (5´-CGATCAGTTAAAARTATWGTAAT-3´) (see HUELSKEN & al. 2006, 2008). PCR products were separated by 2% agarose gel electrophoresis and were purified using the JETSORB Gel Extraction Kit (Genomed, Löhne, Germany) followed by precipitation with 1/10 vol. 3M NaCl and 2.5 vol. of ice-cold 100% EtOH. A comparison of the extracted DNAs is shown in Figure 2 (upper part).

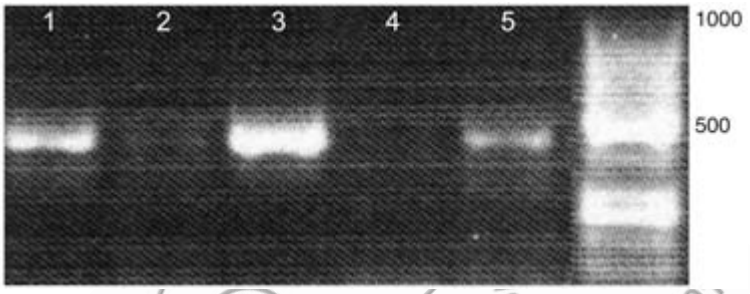

Fig. 3. COI amplification from dried gastropod tissue as revealed on an ethidium bromide-stained agarose gel. 1. *Neverita josephinia*. 2. *Natica fulminea*. 3. *Natica multipunctata*. 4. *Natica acinonyx*. 5. Dried egg mass of *Neverita josephinia*.

Additionally, we have tested our "combination method" for DNA extraction on dried ("mummified") naticid tissues (Fig. 3). Specimens of *Natica fulminea* (GMELIN 1791), *Natica multipunctata* BLAINVILLE 1825, and *Natica acinonyx* MARCHE-MARCHAD 1957 were analyzed where the specimens had been stored for well over 10 years after they initially had been fixed in 90% ethanol and then allowed to dry with the animals retained in the shells. A specimen of *Neverita josephinia* RISSO 1826 and an egg mass of *Neverita josephinia* were dried on purpose for this study. The most important factor in the isolation of adequate amounts of high quality DNA from dried tissue was the incubation time in ATL buffer (Qiagen). Between 48 and 72 hours at 56 °C were required until most of the dried tissue was solubilized (see also CHASE & al. 1998). Non-solubilized material was pelleted at 4.000 xg for 1 min and discarded.

Tissue preservation

Additionally, we evaluated the reliability of DNA amplification from total DNA extracted from naticid tissues that had been stored in different preservatives during field work (ethanol p.a. (EtOH): 100%, 75%, 55%, 35%; formalin: 10% - with 4% formaldehyde; isopropyl alcohol p.a. (IPA): 95%; methanol p.a. (MeOH): 95%; RNAlater™ (Qiagen, Hilden, Germany); Sambuca: 42% (vol.; Molinari, Italy; see Table 1).

Table 1. Amplification success of the COI gene fragment extracted from tissues treated with different preservatives. Except for formalin-fixed tissues, high amounts of DNA could be extracted from each tissue after any preservation duration tested (dof = days of fixation, see Fig. 4).

No.	Treatment	DNA yield	dof	PCR No.			Average
				1	2	3	
1	RNAlater	high	15	1	1	1	1.00
		moderate	25	1	0	1	0.67
		high	134	0	1	0	0.33
2	100 % EtOH	high	15	1	1	1	1.00
		moderate	25	1	1	1	1.00
		high	134	0	1	0	0.33
3	75 % EtOH	high	15	1	1	1	1.00
		high	25	1	1	1	1.00
		high	134	0	0	0	0.00
4	55 % EtOH	high	15	1	1	1	1.00
		high	25	1	1	1	1.00
		high	134	0	0	0	0.00
5	35 % EtOH	high	15	1	1	1	1.00
		high	25	1	0	0	0.33
		high	134	0	0	0	0.00
6	95 % MeOH	moderate	15	1	1	1	1.00
		high	25	1	1	1	1.00
		high	134	0	0	0	0.00
7	95 % IPA	high	15	1	1	1	1.00
		high	25	0	0	0	0.00
		high	134	0	0	0	0.00
8	Formalin	low	15	0	1	0	0.33
		?-low	25	0	0	0	0.00
		?-low	134	0	0	0	0.00
9	Sambuca 42 %-vol	moderate	15	1	0	1	0.67
		high	25	1	1	1	1.00
		high	134	1	1	0	0.67

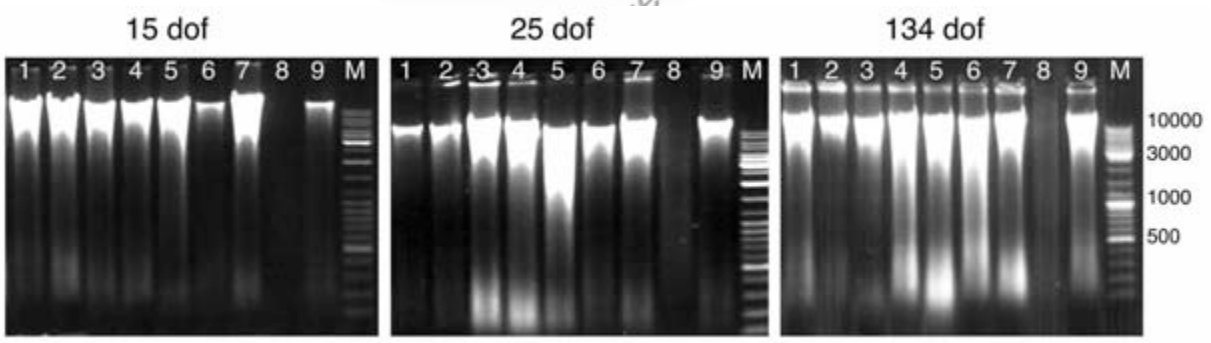

Fig. 4. Ethidium bromide-stained agarose gels with total DNA extracted by the "combination method" from naticid tissue (*N. hebraeus*) after 15, 25, and 134 days of fixation (dof). 1. RNAlater (Qiagen) 2. 100% EtOH p.a. 3. 75% EtOH p.a. 4. 55% EtOH p.a. 5. 35% EtOH p.a. 6. 100% MeOH p.a. 7. 100% IPA p.a. 8. Formalin (4% formaledyde). 9. Sambuca 42%- (vol.) EtOH (Molinari). M. Molecular weight marker.

The preservatives were chosen in order to compare the most commonly used solutions for long-term conservation as well as preservatives that can be applied effectively during field work. Fresh foot tissue was taken from a specimen of *Naticarius hebraeus* (MARTYN 1786) that had been collected in the Bay of Campese (Isola del Giglio, Tuscany, Italy). The sample was cut into 27 pieces of identical size (~ 10 mg), washed with 1x PBS (phosphate-buffered saline), and dried. Samples were stored in

triplicate in 1.5 ml of each preservative for 15, 25, and 134 days (dof = days of fixation) before DNA was extracted using the "combination method" described above (Fig. 3). Each of the 27 samples was amplified three times to calculate an average amplification success (81 PCRs). To check the accuracy of the PCR products the amplicons were digested with the restriction endonuclease HindIII which produced 332 bp and 122 bp fragments as expected from the COI sequence of *N. hebraeus* available in GenBank (EU332643; HUELSKEN *& al.* 2008). Exemplary digestions are shown in Figure 5.

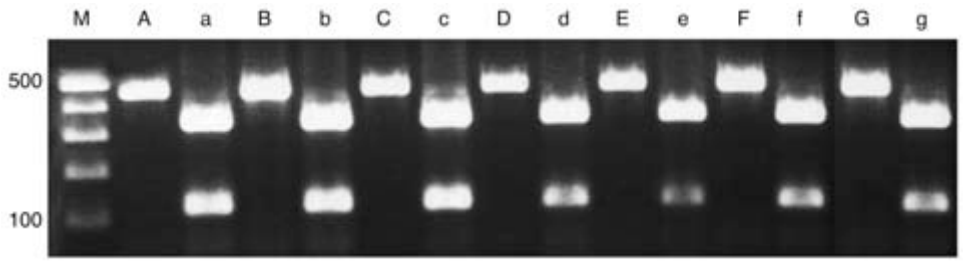

Fig. 5. Exemplary restriction analyses of the amplified COI gene fragments after 15 dof. Capital letters: Uncut gene fragments; lower case letters: gene fragments cut with *Hind*III (332 bp, 122 bp). A/a. RNAlater B/b. 100% EtOH. C/c 55% EtOH. D/d. 35% EtOH. E/e. 95% MeOH. F/f. 95% IPA. G/g. Sambuca 42%- (vol.) EtOH. M. Molecular weight marker.

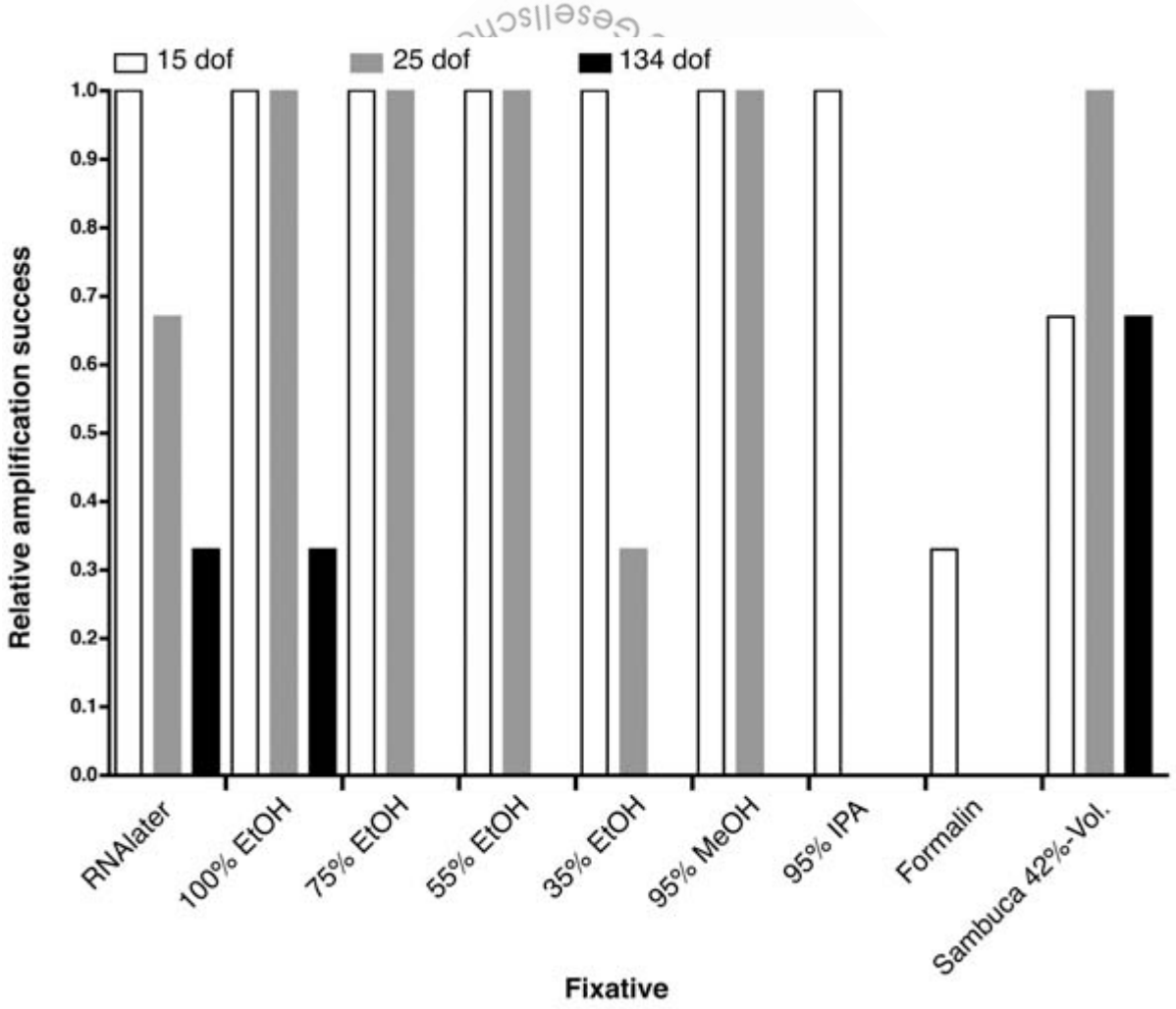

Fig. 6. Dependency of the COI amplification success on the nature of, and storage duration in, a given preservative, tested for 15, 25, and 134 days of fixation (dof). Each bar represents the average amplification success based on three PCRs performed for each sample.

Results and discussion

DNA extraction

Molluscan tissue represents a material that is surprisingly difficult to prepare for reliable and efficient gene fragment amplification and sequence analysis. Amplification success is not guaranteed even when total DNA is successfully extracted in large amounts (SKUJIENÉ & SOROKA 2003, WILLIAMS 2007). In this study we compared the amplification success of two widely used DNA extraction methods and found that a combination of both methods is superior to either of the methods used on its own. The amount of total DNA isolated was largest using the DNeasy Blood and Tissue Kit alone (Fig. 1C), followed by the kit/CTAB combination method (Fig. 1A) and the CTAB method alone (Fig. 1B). By contrast, the highest amplification success rate for the COI gene fragment was obtained with the kit/CTAB combination method (5/5, 100%, >100 ng/PCR), followed by the CTAB method (4/5, 80%, 40-80 ng/PCR). The lowest amplification success rate was obtained when DNA was extracted with the DNeasy Blood and Tissue Kit alone (2/5, 40% <50 ng/PCR).

During CTAB purification of DNA that had been extracted using the DNeasy Blood and Tissue Kit, we observed a white substance at the interphase between the chloroform and water phases. This may suggest the existence of inhibitory substances that were retained on the spin columns of the DNA extraction kit and were eluted together with the DNA. These substances appear to have inhibitory effects on the PCR. The subsequent CTAB purification appears to remove those substances and consequently allows a successful and/or much more efficient PCR. This method therefore combines the advantages of both extraction methods: the kit provides high amounts of DNA (Figs. 1C) as well as uniform and reproducible DNA extraction success, while CTAB purification of the DNA facilitates removal of mucopolysaccharides and inhibitory substances (Fig. 1A). This CTAB purification step can be easily performed even after long-term storage of extracted, low quality DNA and therefore may help in cases of otherwise unsequenceable DNA samples.

Recently, WILLIAMS (2007) reported on a combination of protocols to isolate DNA from littorinids. Contrary to the protocol described by WILLIAMS (2007), we did not use phenol during DNA purification, as it is hazardous to handle and can act as an inhibitor in PCR reactions. The kit/CTAB extraction method presented here has successfully been used in our laboratory for a large number of different naticid species of extremely variable preservation status and reproducibly resulted in sequenceable DNA.

Beside alcohol-fixed tissues, the COI gene fragment was successfully amplified from four of five samples of mummified specimens (Fig. 2, lanes 1-3, 5). Interestingly, while the amount of DNA isolated from *N. fulminea* was the lowest of all specimens analyzed (see Fig. 2, lane 2), it could still be successfully sequenced. This demonstrates that the total amount of extracted DNA does not necessarily correlate with the successful amplification of gene fragments. In previous analyses, we could also show the successful application of the kit/CTAB extraction method to naticid egg masses collected for barcoding analyses (HUELSKEN & al. 2008).

Using the "combination protocol" described above, we demonstrated reliable PCR amplification of mitochondrial COI and 16S gene fragments as well as nuclear gene framents such as a histone H3 gene fragment, a 18S rRNA gene fragment, and a calmodulin gene intron fragment. All these fragments could be amplified from ethanol-preserved tissues, mummified specimens, and egg masses (see HUELSKEN & al. 2006, 2008). Results from dried tissue, however, must be interpreted with caution due to post-mortem changes in nucelotide bases (deamination) that influence sequence reliability (see BOYLE & al. 2004). Despite this caveat, the sequences from our dried samples did not show any sign of alteration. Hence, our kit/CTAB combination method appears to be the optimal DNA extraction method when working with both fresh and dried mucus-rich naticid tissue when compared to the CTAB or kit extractions alone (Fig. 1).

Tissue preservation

In addition to an optimized DNA extraction method, tissue preservation and quality of the storage solution are important factors in obtaining suitable amplification-grade DNA. Except for formalin-preserved tissue (Fig. 3, No. 8), large amounts of total DNA could be successfully extracted from all tissues of different preservation methods and at any storage duration tested (see Fig. 3, after kit

extraction). While DNA extraction was largely storage time- and storage solution-independent, amplification success was more variable and declined over time (see Fig. 5, Table 1). Generally, there were no differences between the amplification success after 15 dof and that after 25 dof, but there were significant differences between both 15 dof and 25 dof specimens and 134 dof samples. Apart from the tissue preserved in formalin, COI sequences were amplified from tissue samples stored in each of the various preservatives tested after 15 and 25 dof. After 134 dof, COI was only successfully amplified from DNA extracted from tissues stored in Sambuca (which contains 42% EtOH), 100% EtOH, as well as RNAlater™ (in order of decreasing amplification success).

However, reliable PCR amplification was also observed for DNA from tissues stored for only a couple of weeks in preservatives such as 75% EtOH, 55% EtOH, 95% MeOH, 95% IPA, and even 35% EtOH. Based on the present study, diluted EtOH as well as other alcohols can therefore be used for short-term preservation of gastropod tissue intended for molecular analyses while they should not be used for long-term storage. The reliability of amplification of the COI gene fragment from gastropod tissue depends strongly on how samples have been treated during collection and storage, supporting earlier analyses with similar results (BOYLE & al. 2004; DAWNWAY & al. 2007). Based on the solutions tested here, the best fixative to preserve mucus-rich naticid tissue are 100% ethanol, Sambuca, and RNAlater™, since reliable amplification of a mitochondrial COI gene fragment was observed after 15, 25, and 134 dof.

The good fixation characteristics of these solutions may be based on the reduction of nuclease activity. Dehydration (EtOH) and high salt concentrations (RNAlater) minimize nuclease activity that would degrade nucleic acids (TOWNSON & al. 1999) and thus allow recovery of intact DNA even after long-term storage. We can only speculate about the good fixation characteristics of Sambuca. This liquor contains high concentrations of sugar, water, and (only) 42% EtOH. It has been demonstrated that high concentration of sucrose drives osmotic dehydration (GIRALDO & al. 2002) and therefore supplements the dehydrative effect of EtOH. A concentrated sugar solution containing 42% EtOH therefore may represent a suitable and cost-efficient field preservative that needs to be further investigated.

In summary, our data indicate that reliable PCR amplification of the COI gene fragment from naticid tissue depends on the DNA extraction method, the storage media, and the duration of conservation. Our results provide guidelines to researchers interested in molecular systematics and evolutionary questions of gastropoda and should help to improve success rates in obtaining sequenceable DNA.

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