Identification of Six Nassarid Snails Using *COI*-Based Restriction Fragment Length Polymorphism

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IDENTIFICATION OF SIX NASSARID SNAILS USING *COI*-BASED RESTRICTION FRAGMENT LENGTH POLYMORPHISM

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ABSTRACT The marine gastropod Nassariidae is a group of edible mud snails that are distributed worldwide. Some of the nassarid species could accumulate algal toxins that cause paralytic shellfish poisoning when being consumed. Therefore, efficient classification of nassarids is important for seafood safety, yet quite challenging because of their complex morphological diversity. In this study, an approach based on cytochrome c oxidase subunit 1–restriction fragment length polymorphism was developed to discriminate six common nassarid species, including *Nassarius nodifer*, *Nassarius conoidalis*, *Nassarius sinarus*, *Nassarius succinctus*, *Nassarius variciferus*, and *Reticunassa festiva*. Specific 709-bp fragments were polymerase chain reaction–amplified, which yielded differential digestion patterns after *MspI* restriction, allowing unambiguous discrimination of all six targeted species. Compared with a previously published approach for determination of nassarids using DNA barcodes, the approach developed here is much more efficient and cost saving, and will be an effective way to detect paralytic shellfish toxins–accumulating nassarids in seafood market.

KEY WORDS: identification, Nassariidae, paralytic shellfish poisoning, RFLP

INTRODUCTION

The mud snail Nassariidae are distributed worldwide on soft bottoms, with most species inhabiting intertidal regions from 0 to 300 m. The species diversity of nassarids is the highest in tropical regions, particularly the Indo-Pacific region (Cernohorsky 1972). In Asia, nassarids are consumed as seafood. Some nassarid species have been reported to contain algal toxins, especially paralytic shellfish toxins (Li & Chen 1981, Lin et al. 1998, Choi et al. 2006) and amnesic shellfish toxins (Goldberg 2003). These nassarid species have been widely recognized as a vector for paralytic shellfish poisoning (PSP) (Basti et al. 2018). Thus, consumption of such nassarids often leads to poisoning incidents in Asian countries, especially in China (Qin & Yu 2003, Shui et al. 2003, Yu et al. 2007, Luo et al. 2008, Zhang et al. 2009). Therefore, proper identification of such poisonous nassarids is necessary.

Identification of nassarid species is mainly based on shell morphology, especially the sculpture (Haasl 2000). The use of morphological characters for nassarid identification, however, is sometimes difficult because of intraspecific variation (Li et al. 2010). For example, the eurythermic species *Nassarius variciferus* (Adams, 1851) showed high intraspecific morphological diversity in the presence or absence of varices on body whorl and the color of spiral bands (Zou et al. 2012). Furthermore, interspecific convergence may also pose difficulties in species identification (Bargues & Mas-Coma 1997).

Molecular methods have been widely developed for species identification in the past two decades. The use of DNA-based techniques can overcome the drawbacks of morphological identification to a large extent (Tautz et al. 2003). Some examples of such methods include randomly amplified polymorphic DNA (Bardakci & Skibinski 1994), amplified fragment length polymorphism (Congiu et al. 2002), DNA barcoding (Hebert et al. 2003a), and polymerase chain reaction (PCR)–based restriction fragment length polymorphism (RFLP) (Wolf et al. 1999).

Among these methods, PCR-RFLP relies on analysis of DNA fragment patterns after treatments with restriction enzymes and, therefore, is easy, rapid, and cost-effective. This method has been widely used for meat and fish identification, commonly using conserved mitochondrial (mt) DNA. Mitochondrial DNA presents a higher copy number and faster rate of mutation, making it generally more appropriate in the study for species identification

Zhanjiang (Guangdong Province of China), six *N. conoidalis* were collected from a local market in Dongshan County (Fujian Province of China), and six *N. sinarus* were collected from a local market in Zhoushan (Zhejiang Province of China). Other species, including *N. variciferus*, *N. succinctus*, and *R. festiva* (two individuals from each location), were collected from Binzhou (Shandong Province), Qingdao (Shandong Province), and Lianyungang (Jiangsu Province of China).

Specimens were stored in 95% ethanol. Genomic DNA was extracted from small pieces of foot tissue by the CTAB method as modified by Winnepenninckx et al. (1993).

A ca COI G F a

Universal *COI* primers (LCO1490: 5′-ATT CAA CCA ATC ATA AAG ATA TTG G-3′ and HC02198: 5′-TAA ACT TCT GGA TGT CCA AAA AAT CA-3′) were used for PCR amplification of all samples (Folmer et al. 1994). The standard PCRs were performed in a total volume of 25 µL with 0.5 µL of template DNA (approximately 100 ng), 2.5 µL of 10× PCR buffer (Mg^{2+} plus), 0.5 µL of dNTPs (10 mM), 1 µL of each primer (10 µM), and 0.25 µL (5 U/µL) of *Taq* DNA polymerase. The following profile was used: predenaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 52°C annealing temperature for 30 sec, extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. Generated PCR products were checked by electrophoresis on 1.5% agarose gel with ethidium bromide in 0.5× Tris Borate EDTA and were photographed under ultraviolet light using the gel documentation system.

DNA S c a S c R c E

After PCR amplification, each product of *Nassarius nodifer*, *Nassarius conoidalis*, *Nassarius sinarus*, *Nassarius succinctus*, *Nassarius variciferus*, and *Reticunassa festiva* was sequenced by BGI sequencing, Beijing, China. These sequences were completed by retrieval from the corresponding mt genome, which were conducted in a previous analysis (unpublished) and submitted to GenBank with accession numbers KY783915–KY783920. The restriction enzymes *MspI* and *TaqI* were selected for the sequenced nucleotide using software Primer Premier 5 (Lalitha 2000). Gene alignment, recognition sites of two endonuclease, and expected product sizes are shown in Figure 1 and Table 1.

P a C a R ac -R c F a L P A a

Digestion was performed with restriction enzymes *MspI* and *TaqI*. Digestion reactions were carried out in a total volume of 20 µL containing 5 µL of PCR product, 1 µL of enzyme (10 units/µL), 2 µL of 0.1% BSA, and 2 µL of 10× *TaqI* buffer for *TaqI* or 2 µL of 10× T buffer for *MspI*, respectively. The final volume was made up to 20 µL with autoclaved sterile dH_2O. Restriction reactions were conducted in an incubator for 4 h at either 65°C for *TaqI* or 37°C for *MspI*. Digested products were run on 1.5% agarose gels and electrophoresed with 1× Tris acetate EDTA at 90 V for 45 min and visualized under ultraviolet light. A 100-bp DNA ladder was used as the reference marker.

RESULTS

A 709-bp fragment was amplified using universal *COI* primers and no size variations were found among different samples. For the analysis of digested products, only major bands were considered. The results showed that *TaqI* restricted positively in all samples (Fig. 2A, B), but failed to identify *Nassarius succinctus*, *Nassarius sinarus*, and *Nassarius conoidalis* because of the same length of restriction products. With respect to *MspI*, although it failed to restrict for *Reticunassa festiva*, all six nassarids exhibited distinctive restriction products (Fig. 2C, D). The length of the restriction products was in accordance with the size expected from the nucleotide sequences (Table 1).

DISCUSSION

Using only *Msp I* was sufficient to generate species-specific digested products to reveal levels of *COI* variation. According to Mueller et al. (2015), a mutation which affected an enzyme recognition site would lead to variations within samples of the same species; in the present analysis, no polymorphisms were detected when either *Taq I* or *Msp I* was applied to multiple samples of each species. This could be because of the conservation of mt *COI* gene within each species.

The DNA barcoding method has been proven as a useful tool for species identification in many animal and plant groups (Hebert et al. 2003a, 2003b). As for nassarid gastropods, Zou et al. (2012) reported a barcoding analysis by integrating mt and nuclear sequences and morphological characters, and successfully identified the species under study. Although this DNA sequencing–dependent technique was quite accurate and reliable, it was slow, costly, and unavailable for routine food species identification (Girish et al. 2005). Compared with DNA barcodes, PCR-RFLP was much more economical, easier, and faster and did not require high-quality or a large amount of DNA (Haider 2003). In addition, the materials and equipment, such as restriction enzymes, water bath, and electrophoresis system, were quite accessible for most biotechnology laboratories.

Previous studies using the PCR-RFLP method have been reported on the identification of livestock (Girish et al. 2005, Haider et al. 2012), fish (Mueller et al. 2015, Ferrito et al. 2016), microorganisms (Guillamón et al. 1998), and shellfish (Fernández-Tajes et al. 2011). Among these studies, many were related to fraudulent substitutions or adulterations of livestock meat or fish products. The present study focused on the identification of marine nassarids that may contain paralytic shellfish toxins. The toxins-containing species of nassarid in the China Sea have been discussed previously (Shui et al. 2003, Yu et al. 2007, Zhang et al. 2009). According to their results and records, most of the poisoning incidents via the consumption of nassarids were related to *Nassarius succinctus*, *Nassarius sinarus*, *Nassarius conoidalis*, and *Nassarius nodifer*. With respect to *Nassarius variciferus* and *Reticunassa festiva*, nonpoisoning accidents were found or recorded in these two species, and the widespread *N. variciferus* is still accessible in most seafood markets in China. In mainland China, about 30 species of nassarids have been reported (Yang & Zhang 2009). In the future, with more DNA data being sequenced, this method may be applied in the identification of PSP- versus non-PSP–accumulating species of nassarids.

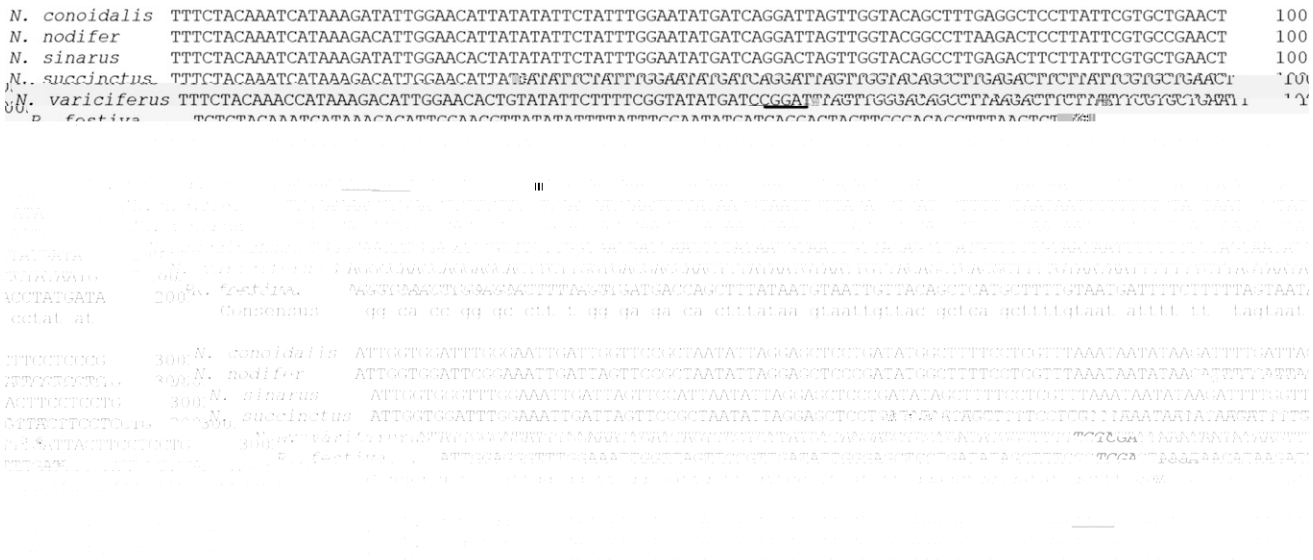

Figure 1. Partial nucleotide sequences of the mt *COI* gene used for the RFLP analysis of six nassarid species. Recognition sites of *M I* (C˄CGG) are underlined, whereas those of *Ta I* (T˄CGA) are in italics and bold style.

It can be concluded that the six nassarid species can be qualitatively identified and differentiated by the quick, easy, low-cost, and reliable PCR-RFLP of the mt *COI* gene. This method could also be a potential way for future monitoring of PSP-accumulating nassarid species in the seafood market.

TABLE 1.

Expected size (bp) of digested DNA fragments ondigestion with two restriction enzymes.

	Species					
Enzyme	***R c a a a***	***Na a a c***	***Na a cc c***	***Na a***	***Na a a***	***Na a c a***
MspI	Negative	55, 63, 70, 521	315, 394	67, 642	63, 70, 576	109, 240, 360
TaqI	72, 184, 190, 263	95, 263, 351	95, 614	95, 103, 511	95, 614	95, 614

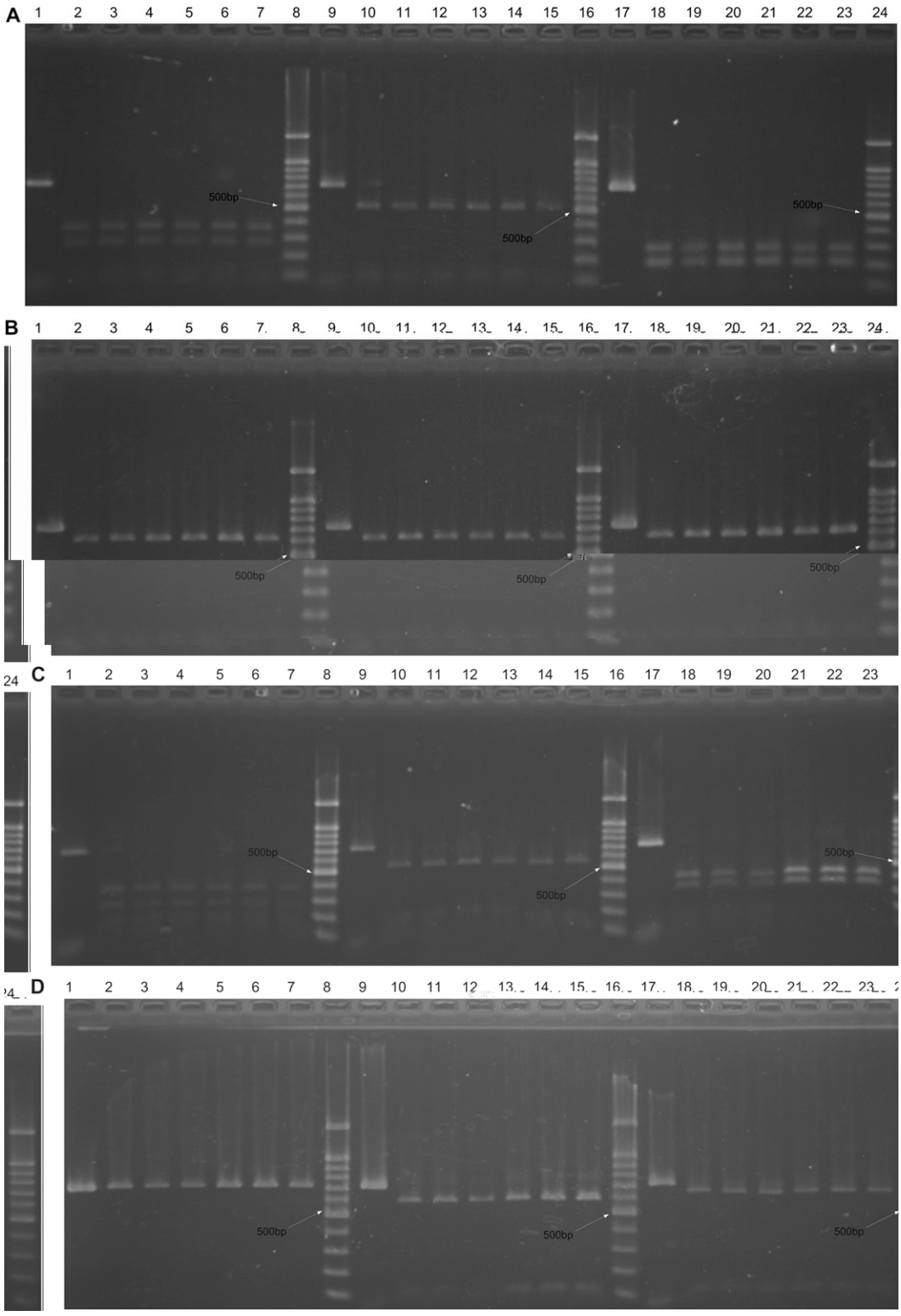

Figure 2. Restriction profiles of *COI* amplicons with two restriction enzymes in the nassarid samples analyzed. Lanes 8, 16, and 24, 100-bp DNA ladder. (A) *Ta I*. Lanes 1, 9, and 17, unrestricted PCR product of *Na a a c* , *Na a* , and *R c a a a*, respectively; lanes 2–7, 10–15, and 18–23, restriction profiles generated by *N. a c* , *N.* , and *R. a*, respectively. (B) *Ta I*. Lanes 1, 9, and 17, unrestricted PCR product of *Na a cc c* , *Na a C a* , and *Na a a* , respectively; lanes 2–7, 10–15, and 18–23, restriction profiles generated by *N. cc c* , *N. c a* , and *N. a* , respectively. (C) *M I*. Lanes 1, 9, and 17, unrestricted PCR product of *N. c a* , *N. a c* , and *N. cc c* , respectively; lanes 2–7, 10–15, and 18–23, restriction profiles generated by *N. c a* , *N. a c* , and *N. cc c* , respectively. (D) *M I*. Lanes 1, 9, and 17, unrestricted PCR product of *R. a*, *N. a* , and *N.* , respectively; lanes 2–7, 10–15, and 18–23, restriction profiles generated by *R. a*, *N. a* , and *N.* , respectively.

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