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NON-INVASIVE METHOD TO OBTAIN DNA FROM FRESHWATER MUSSELS (BIVALVIA: UNIONIDAE)

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ABSTRACT To determine whether DNA could be isolated from tissues obtained by brush-swabbing the mantle, viscera and foot, mantle-clips and swabbed cells were obtained from eight *Quadrula pustulosa* (Lea, 1831). DNA yields from clips and swabbings were 447.0 and 975.3 ηg/μL, respectively. Furthermore, comparisons of sequences from the ND-1 mitochondrial gene region showed a 100% sequence agreement of DNA from cells obtained by clips and swabs. To determine the number of swabs needed to obtain adequate yields of DNA for analyses, the visceras and feet of 5 *Q. pustulosa* each were successively swabbed 2, 4 and 6 times. DNA yields from the 2, 4 and 6 swabbed mussel groups were 399.4, 833.8 and 852.6 ng/μL, respectively. ND-1 sequences from the lowest yield still provided 846–901 bp for the ND-1 region. Nevertheless, to ensure adequate DNA yield from cell samples obtained by swabbing, we recommend that 4 swab-strokes of the viscera and foot be obtained. The use of integumental swabbing for collection of cells for determination of genetic relationships among freshwater mussels is noninvasive, when compared with tissue collection by mantle-clipping. Therefore, its use is recommended for freshwater mussels, especially state-protected or federally listed mussel species.

KEY WORDS: Unionidae, genetics, integument, swabbing, mantle-clipping

INTRODUCTION

The use of mantle-clipping for biopsy has become a common technique for collection of tissues from unionoid mussels for genetic analyses (Buhay et al. 2002, Eackles & King 2002, Jones et al. 2004, Curole et al. 2004, Campbell et al. 2005, Geist & Kuehn 2005, Grobler et al. 2006). Berg et al. (1995) observe no significant differences in mortality rates in mantle-biopsied versus nonbiopsied muckets, *Actinonaias ligamentina* (Lamarck, 1819) and mapleleafs, *Quadrula quadrula* (Rafinesque, 1820). However, mantle-clipped snuffboxes, *Epioblasma triquetra* (Rafinesque, 1820), showed a mortality rate of 56.3% ($n = 16$) after 1.5 y of postbiopsy observation at the Aquatic Wildlife Conservation Center (AWCC) (Eckert, N., VDGIF, Marion, Virginia, pers. comm.). Although mortality of mantle-clipped mussels may not be attributed directly to tissue removal, inspection of the dead *E. triquetra* showed regression of the nacre and shell deformity in the valve locations where mantle edges were removed (Fig. 1). Because mantle biopsy is an invasive procedure that may induce mortality, its use on federally endangered mussel species is a questionable procedure for genetic analyses. The goal of this study was to determine whether a procedure less invasive than mantle biopsy is available for collection of DNA for genetic analyses. Our objective was to determine whether viable DNA could be obtained by integumental swabbing from pimplebacks, *Quadrula pustulosa* (Lea, 1831) and to confirm mDNA sequence agreement among tissues obtained from swabbing and mantle-clipping from the same mussels.

MATERIALS AND METHODS

Tissue Collection

Tissues were obtained from *Q. pustulosa* at the Freshwater Mollusc Conservation Center, Virginia Tech, Blacksburg, Virginia ($\bar{\chi} = 70.7$ mm, $s = \pm 11.7$). To test the feasibility of isolating DNA by swabbing of the viscera, foot and mantle, we initially sampled both mantle clips and brush swabs from 8 *Q. pustulosa.* Clips (approximately 3 × 5 mm) were taken from the edge of the mantle and stored in 95% ethanol. Cell samples were taken by using approximately 8 vigorous strokes with a bristle brush (CYB-1; Gentra Systems, Minneapolis, Minnesota). Strokes covered all structures within the visceral cavity, including the mantle surface, viscera and foot. Brush tips were stored in lyses buffer. Then, we determined (1) the amount of DNA that can be obtained using a bristle brush, compared with conventional mantle clips; (2) whether ND-1 could be amplified from the DNA and (3) whether ND-1 sequences amplified using buccal swabs was identical to results from mantle clips.

Because excessive scrubbing with bristle brushes resulted in some disruption of the mantle, we also investigated a collection technique using the viscera and foot only. Furthermore, it would be informative to determine the minimum number of integumental swabs required to provide an adequate amount of mDNA for sequence analyses. For this objective, we sampled an additional 15 individuals of *Q. pustulosa*, using 6 passes of the brush on each of 5 individuals, 4 passes on another 5, and 2 passes on the 5 remaining mussels. Care was taken to rotate the brush between strokes and thus present a clean surface for collection at each stroke. After determining the amount of DNA obtained using each number of strokes, the ND-s gene region was sequenced from the least invasive technique (2 strokes) to test the feasibility of using this as a source of DNA for sequencing reactions.

DNA Extraction

DNA was extracted from all samples using the Purgene DNA extraction kit. For extraction from brushes, the protocol described by Purgene was modified by increasing the initial amount of lyses buffer used from 300 μL to 450 μL, to ensure complete coverage of brush-bristles in a 1.5 mL Eppendorf tube. DNA was extracted

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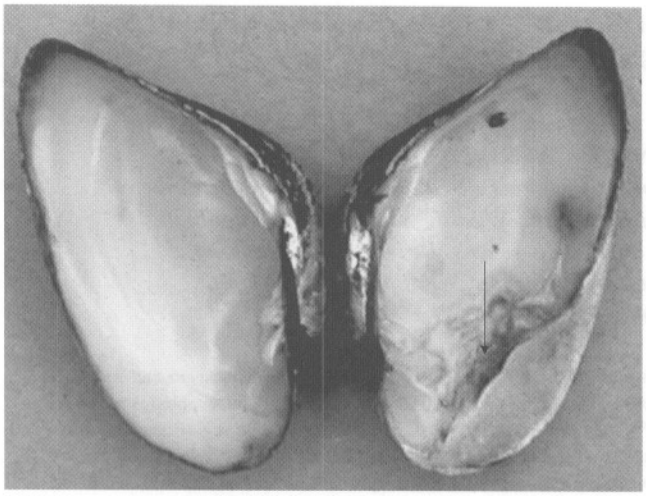

Figure 1. Shell of *Epioblasma triquetra* showing regression of nacre in the location of mantle edge removal (arrow), following postmantle clipping observation for 1.5 y.

from mantle-clipped tissue following the standard Puregene protocol for solid tissue.

Molecular Analysis

We used the NADH dehydrogenase (ND-1) mitochondrial gene region to verify that DNA obtained from swabs was indeed mussel DNA and that it was suitable for sequencing reactions. Primer sequences and polymerase chain reaction (PCR) amplification conditions were as reported by Buhay et al. (2002) and Serb et al. (2003). Primer sequences were forward: 5′-TGGCAGAAAAGTGCATCAGATTAAAGC-3′; reverse 5′-CCTGCTTGGAAGGCAAGTGTACT-3′. PCR reaction mixtures contained 100 ng of genomic DNA, 1× PCR buffer, 4.0 mM $MgCl_2$, 0.4 mM dNTPs, 1.0 μM of each primer and 1.5 U Ampli*Taq* Gold DNA polymerase, with ddH_2O added to a total volume of 25 μL. The thermal cycler profile consisted of an initial 95°C for 8 min; followed by 35 cycles of: 94°C for 40 sec, 50°C for 60 sec and 72°C for 90 sec; with a final extension step at 72°C for 2 min; and a final hold at 4°C.

PCR products were purified using a Qiagen DNA Purification kit and were sequenced using Applied Biosystems Big Dye v.3.1. Reaction mixtures consisted of 30 ng PCR product, 5-μM primer, 2 uL Big Dye 3.1 (diluted 1:1), with ddH_2O added for a final volume of 7.5 uL. The cycle reaction consisted of 30 cycles, each of 94°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. The products were cleaned by centrifuging through hydrated Sephadex (Sigma) in Millipore filter plates, dried down and resuspended in Hi-Di Formamide (Applied Biosystems). The samples were denatured at 95°C for 5 min, and cooled to 4°C for 2 min before being loaded on the Applied Biosystems DNA Analyzer 3730 for processing. SEQUENCHER software (ver. 4.11) was used to align and edit sequences from stored GENESCAN files.

RESULTS

The average DNA yields obtained varied by collection technique (Table 1). The standardized collection method from mantle clips yielded an average of 447.0 ng/μL. Eight vigorous passes of the bristle brush over the viscera and mantle yielded an average of 975.3 ng/μL (with high deviations in yield), whereas swabs of the viscera and foot yielded 852.6, 833.8 and 399.4 ng/μL using 6, 4 and 2 strokes, respectively (and with lower standard deviations compared with strokes involving the mantle). During DNA isolation, the amount of protein in mucous-derived samples was very high, and care had to be taken to ensure full precipitation of proteins before continuing with the isolation procedure. It was necessary to repeat the protein precipitation stage of the Purgene procedure in some instances.

Sequences for the ND-1 gene region obtained from mantle clips and integument swab samples from the same three individuals aligned with 100% accuracy (847–895 basepairs; Genbank accession numbers DQ640237 to DQ640239). Sequences of ND-1 for DNA isolated after 2 strokes of the bristle brush yielded a clean 846–901 bp for the ND-1 region in two random samples (Genbank accession numbers DQ640240 and DQ640241).

DISCUSSION

Comparison of our data shows that swabbing of the foot and viscera of *Q. pustulosa* is a reliable method for collection of cells for DNA analyses. The DNA obtained from the swabbing technique was pure mussel DNA; our analyses show that it is of high quality, with 100% sequence alignment with DNA obtained from mantle clips from the same mussel specimens. The DNA yield from cells collected by swabbing was equal or greater than the yield from mantle clips. Our data show that 2, 4 and 6 swab passes over the foot and viscera provided sufficient quantities of DNA for reliable analyses. Because the DNA yield remains relatively constant with 4 and 6 swabbings (852.6 and 833.8 ng/μL), and then declines with 2 (399.4 ng/μL), we recommend that 4 swabs of the foot and integument be used as a standard method for collection of cells. The number of swabs required to provide sufficient cell samples for analyses may vary by mussel size and species. We recommend that researchers use this swabbing protocol rather than tissue collection by mantle-clipping, because it is noninvasive and provides reliable DNA for genetic analyses. This is especially true for federally listed mussels, typically restricted from intentional sacrifice in federal collection permits.

We observed a high protein yield in the swabbed samples that could possibly interfere with the results of future DNA analyses. We hypothesize that this protein was included in integumental mucous that was sampled inadvertently with swabbed cells. We recommend special care be exhibited during the protein-precipitation step of DNA isolations from swabbed samples.

TABLE 1.

Results of DNA extraction from mantle and integument samples. *n* = number of mussels tested.

Cell Collection Technique	Tissue Source	Number of Swab Strokes	*n*	Mean DNA Yield (ng/μl)
Mantle clipping	Mantle		8	447.0 ± 143.3
Integument swabbing	Viscera and mantle	8	8	975.6 ± 779.5
	Viscera and foot	6	5	852.6 ± 237.4
	Viscera and foot	4	5	833.8 ± 317.0
	Viscera and foot	2	5	399.4 ± 83.5

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