DEVELOPMENT AND UTILIZATION OF GENETIC PROBES FOR STUDYING ZEBRA MUSSEL VELIGERS

Marc E. Frischer [1], Jane A. Wyllie[2], Andrew S. Hansen [2], and Sandra A. Nierzwicki-Bauer[2]

AUTHORS: [1]Skidaway Institute of Oceanography, 10 Ocean Science Circle, Savannah, GA 31411. [2]Rensselaer Polytechnic Institute, Darrin Fresh Water Institute, 110 8th St., Troy, NY 12180

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Abstract. A key issue in the management of zebra mussel populations is early, rapid, and accurate detection of the planktonic forms of the mussel veligers. In this study, an alternative technology to microscopic enumeration of zebra mussel veligers has been developed based on a species specific genetic probe targeting the 18S rRNA molecule. Probe specificity and sensitivity were determined empirically in the laboratory. The probe did not hybridize to any other bivalve representative tested, including a close relative of the zebra mussel, Mytilopsis leucophaeta. A single veliger could be detected by 18S rRNA targeted probes. The probe was tested in three different field settings including the Hudson River, NY, Lake Champlain, NY/VT, and Lake George, NY and compared to standard methods. Results were generally consistent with microscopy methods. A long term goal of this work is the application of genetic probe technology to the development of a field device for the routine detection and quantification of zebra mussel veligers.

INTRODUCTION

The zebra mussel (Dreissena polymorpha) is one of the latest in a long list of exotic species to have been introduced to North America (Mills et al., 1993). This small freshwater clam has been responsible for fouling municipal, electric power generation and industrial water intake facilities disrupting food webs and ecosystem balances; and interfering with sport and commercial fishing, navigation, recreational boating, beach use, and agricultural irrigation throughout North America. Native to the drainage basins of the Black, Caspian and Aral Seas, the zebra mussel was introduced to Europe in the late 1700s. Microscopic zebra mussel larvae called veligers are thought to have been transported from Europe to North America in the bilge water of a trans-Atlantic freighter and introduced into the Great Lakes in 1985 or 1986 (Roberts, 1990; Carlton, 1993; Ludyanskiy et al., 1993).

Since its first introduction to North America, Dreissena polymorpha, has rapidly colonized US and Canadian waters. The current North American range of zebra mussels extends well into Canada and as far South as Louisiana in the Mississippi River. Based on population distributions determined in 1990 and 1992, (New York Sea Grant? DPIR, 1990, 1992), it has been projected that by the year 2000, the zebra mussel will have colonized all North American rivers, lakes, and reservoirs that fit its broad ecological requirements (Ludyanskiy et al., 1993). As of November 1996, no confirmed sightings of zebra mussels in Georgia had been reported. However, risk studies based on water chemistry parameters suggest that a number of environments in Georgia are at high risk of being colonized by zebra mussels, particularly Lake Lanier and the Chattahoochee River (Bolton, in press).

In large part the colonization success of the zebra mussel in North America is due to its method of reproduction and life history. The zebra mussel is unique compared to other freshwater bivalves, except~ perhaps Mytilopsis, in that it produces free-swimming larvae called veligers. It is primarily during this life phase that the species colonizes new environments. Because the veliger is the primary means by which zebra mussels colonize new environments, the detection of veligers in the water before populations become established is a critical component of current management strategies that seek to minimize the impact of established mussel populations and protect pristine environments.

Because of their small size (50 - 200 microns) and paucity of distinguishing morphological features, identification of veligers by standard microscopic methods is a tedious and labor intensive process. Therefore, the development of simple early detection methods that are reliable, sensitive, and can be accomplished with a minimal amount of training and equipment, has been an area of active investigation. Recent advances in molecular biology and biotechnology have allowed for the development of tests that satisfy these criteria. In this paper we describe the development of a zebra mussel-specific molecular probe and its use for studying zebra mussel veligers. This probe should allow researchers, managers, and those concerned with the spread of the zebra mussels the means to rapidly and accurately identify planktonic forms of *D. polymorpha* against a diverse background of other planktonic organisms.

METHODS

Probe Construction

Isolation of genomic DNA from zebra mussels and other bivalves. Adult zebra mussels were collected from the Hudson River near Troy Lock Number One. Total genomic DNA suitable for Polymerase Chain Reaction (PCR) amplification was purified from fresh or frozen adductor muscle as previously described (Frischer and Nierzwicki Bauer, 1995; Wyllie et al., in prep).

Briefly, muscle tissue was homogenized, cellular debris removed, and intact nuclei collected by centrifugation. Nuclei were lysed with SDS, proteins removed by digestion with Proteinase K, and RNA removed by RNase A digestion. DNA was further purified by organic extraction with phenol and chloroform and concentrated by precipitation in ethanol. This method resulted in DNA suitable for PCR amplification.

PCR amplification, sequencing, and phylogenetic construction. Universal 18S PCR primers (Rice, 1990) or primers designed specifically for the amplification of the complete 18S rRNA gene of zebra mussel were utilized. The Rice primers used were 5'-caacctggttgatcctgccagt (forward) and 5'-ctgatccttctgcaggttcacctac (reverse). Primers designed specifically for amplifying the complete zebra mussel gene were 5' ctgccagtagtcatatgc (forward-ZEB-15) and 5' accttgttacgacttttac (reverse-ZEB-1765). Amplification was accomplished in 10 mM Tris (pH 8.3), 50 mM KCl, 0.1% gelatin, 1.7 mM $MgCl_2$ after 40 amplification cycles [94°C (1 min), 55°C (1 min), and 72°C (1.5 min) initiated after a 5 minute denaturation step at 95°C and followed by a 10 minute final extension step at 72°C].

To facilitate sequencing, the 18S rRNA gene PCR product was cloned into the bacterial plasmid sequencing vector pT7Blue (Novagen, Inc., Madison, WI). Similarly, the 18S gene from *Corbicula fluminea* and *Mytilopsis leucophaeta* were cloned into bacterial plasmids. Sequencing was accomplished manually using the Sequenase V 2.0 kit (US Biochem, Cleveland, OH) or by automated sequencing at the University of Maine. Both forward and reverse strands were sequenced to ensure maximum sequence accuracy. Sequence data was compared to secondary structural models of the 18S rRNA gene of *P. mageallanticus*, aligned to a close relative by maximum homology, and aligned manually to the Ribosomal Database Project (RDP; Maidak et al., 1994) alignments using the Genetic Database Environment software package (Smith et al., 1992). DNA distance phylogenies were inferred using the programs available in the PHYLIP version 3.51c package (Felsenstein, 1993). The statistical validity of branching patterns was determined after bootstrap analysis (100 times) and a consensus tree derived.

Probe identification and synthesis. Regions of the 18S rRNA gene unique to the zebra mussel were identified by computer-aided sequence comparisons within the aligned database. Small regions (15-30 base pairs) that exhibited at least 10% difference from all other bivalves were considered as potential probe target sites. Oligonucleotides complimentary to these regions were chemically synthesized for testing as zebra mussel specific probes.

Probe Specificity and Sensitivity

The specificity of each potential probe was tested against purified 18S rRNA PCR gene product and cloned PCR product from several related bivalves at calculated hybridization temperatures (Innis et al., 1990). If a particular probe appeared to be promising, based on its hybridization to zebra mussel genetic material and minimal hybridization to other organisms, the hybridization temperature and wash stringency was optimized empirically. The detection sensitivity of probes were determined against PCR amplified zebra mussel 18S rRNA gene product. Oligonucleotide probes were labeled with digoxigenin-dUTP with terminal transferase following the Genius system oligonucleotide tailing protocol outlined by the manufacturer (Boehringer Mannheim, IN). Hybridization of DNA, rRNA, or extracted water samples immobilized on charged nylon, was also accomplished according to instructions provided by Boehringer Mannheim.

Enumeration of Veligers in Water Samples

Veligers in water samples from 3 different environments were detected/enumerated by standard microscopic techniques and with the molecular probe. Comparisons were made in the Hudson River, NY, Lake Champlain, NYNT, and in Lake George, NY. The Hudson River and Lake Champlain support zebra mussel populations, while Lake George was thought to be pristine with respect to the mussels. Depth integrated water samples (200 L) were pumped using a diaphragm pump through a 44 micron plankton net and concentrated to approximately 60 mls. The concentrate was split and one half fixed in 25% ethanol for microscopy and the remaining 30 mls frozen at -80°C for probe analysis. Duplicate samples were collected at each site. Veligers in ethanol fixed samples were enumerated by cross-polarized light microscopy using standard methods (Marsden, 1992). Frozen water samples were further concentrated by centrifugation at 2300 x g for 10 minutes and the final pellet resuspended in 1 ml of deionized water. RNA was extracted as previously described for veligers, immobilized onto charged nylon, and hybridized to the zebra mussel specific probe ZEB715a. Water samples were collected bi-monthly from the Hudson River near the Troy City Marina, at 4 locations in Lake Champlain 3 times during the 1995 summer, and bi-monthly from Lake George at 7 locations.

RESULTS AND CONCLUSIONS

Molecular Phylogeny

The overall base pair homology of the zebra mussel 18S rRNA gene was 96.3% over a 1666 base pair region compared with its closest sequenced relative, Mytilopsis leucophaeta. Phylogenetic relationships derived from DNA distance analysis were generally consistent with phylogenies derived from morphological characteristics (Figure 1). Despite the overall high homology between the zebra mussel and its close relatives, sufficient divergence existed to define regions suitable for probe targeting.

Probe Design

Thirteen sites were identified for probe targeting. Curiously, after empirical testing of all of these probes, only one (ZEB715a) hybridized strongly to genetic material from *D. polymorpha*, but not to any of the other organisms tested (Figure 2). In most cases, probes other than ZEB715a did not hybridize

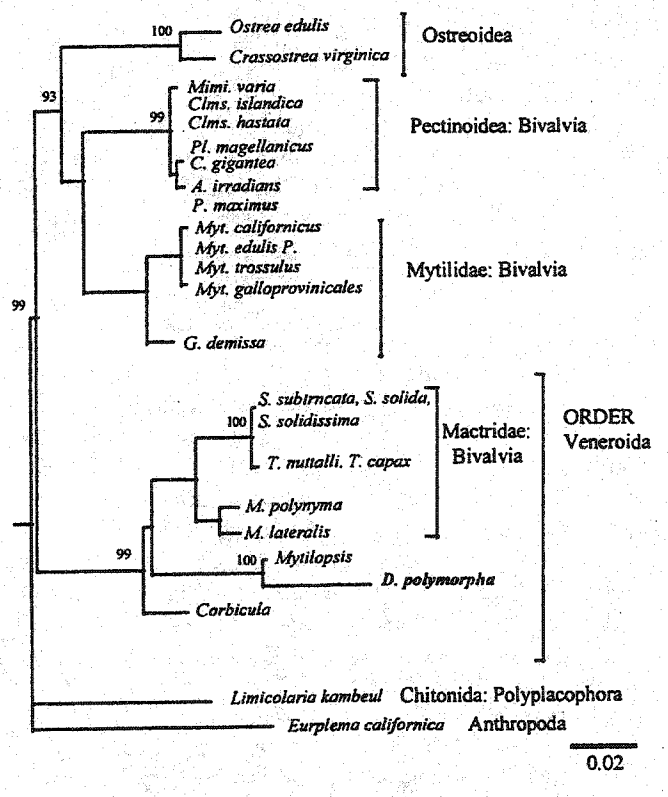

Figure 1. Inferred taxonomic relationship between bivalves and other mollusks derived from 18S rRNA gene sequences. The scale bar indicates 0.02 fixed differences per nucleotide position. Bootstrap values below 75 (out of 100) are not shown.

to any material, including zebra mussel. The sensitivity of the probe was determined by hybridizing radioactively labeled 32P and non-radioactively labeled (digoxigenin) probe to PCR amplified zebra mussel 18S rDNA. Detection levels were between 1 to 10 ng by both labeling methods (Data not shown). All subsequent studies utilized the non-radioactively labeled probe.

Probe Detection Method Optimization

Methods for extraction and detection of rRNA from veligers were developed. Optimal extraction procedures involved heat lysis, physical disruption of the shell by vigorous mixing in the presence of glass bead (100-150 μm), and removal of proteins by digestion with Proteinase K. Heat lysis or physical disruption alone was not sufficient to yield sensitive detection, nor was treatment with Hexa decyl trimethyl ammonium Bromide CTAB. Using the optimized protocol, a single veliger could be detected by the probe (Figure 3).

Detection of veligers from natural waters

Veligers were consistently detected in environments known to support zebra mussel populations, using either probe or microscopy techniques. However, it was unclear whether the intensity of the probe signal was directly correlated with the

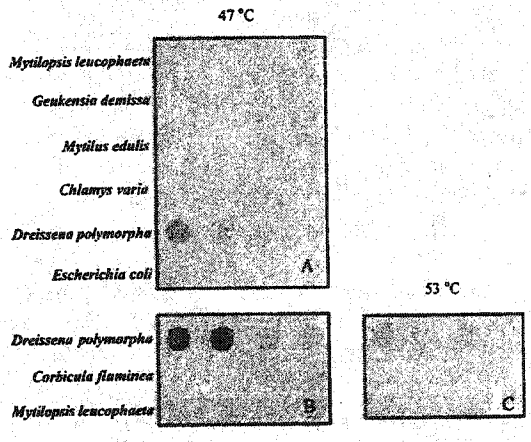

Figure 2. Specificity of probe ZEB715a. Hybridization at 47°C with bacterial clones of bivalve 18S rRNA genes (A), and 18S PCR gene product (B). Raising hybridization temperature to 53°C eliminated non-specific hybridization with *M. leucophaeta* (C).

density of veligers, since in several instances stronger probe signal was observed in samples that contained fewer veligers as determined by microscopic counting (Data not shown). During this study veligers were discovered in Lake George. This was surprising since no adult populations have been observed or reported from this lake. Probe results indicated that, at least on some dates, veligers were present throughout the lake, while they were observed in only a few sites microscopically (Figure 4). From the 21 direct comparisons shown in Figure 4, there was disagreement between 7 samples. In 5 cases the probe detected veligers while none were observed microscopically. In 2 cases, veligers were observed microscopically but no probe hybridization signal was detected. However, ostracods were often abundant in the samples and can easily be mistaken for zebra mussel veligers under cross-polarized light. These results

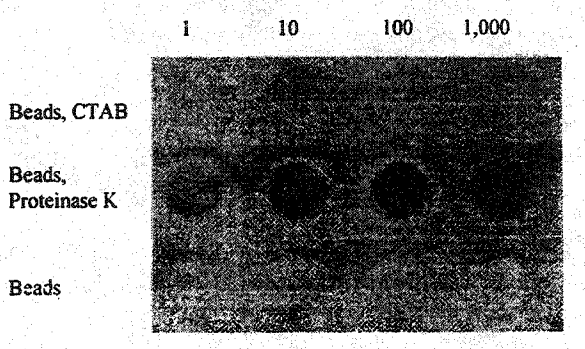

Figure 3. Detection of veligers with 18S rRNA targeted probe following extraction by physical disruption/heat only (Beads), physical disruption/heat plus treatment with Proteinase K, and physical disruption/heat plus treatment with Hexa decyl-trimethyl ammonium Bromide (CTAB). The number of veligers per dot is indicated at the top.

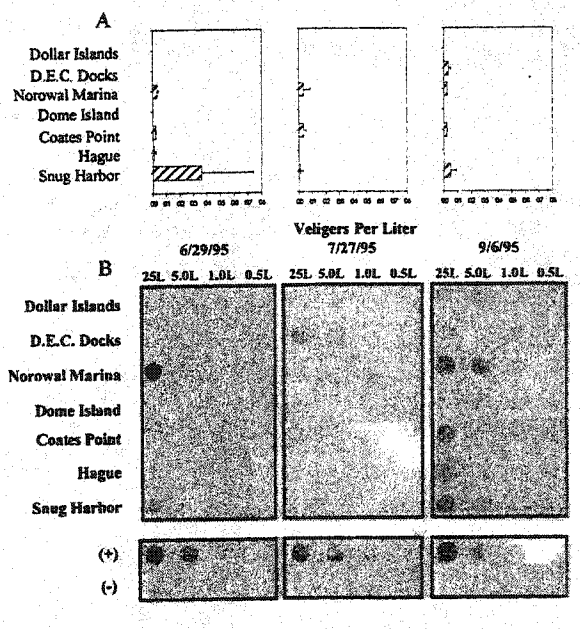

Figure 4. Detection of zebra mussel veligers by cross polarized light microscopy (A) and molecular probe hybridization (B) in Lake George, NY. Microscopic enumerations ale reported as veligers per liter and the amount of water extracted per probe dot is indicated. Positive and negative hybridization controls are DNA from zebra mussels or *E. coli* to control for non specific hybridization, respectively.

suggest that the probe can be used to detect the presence of zebra mussel veligers in natural waters and may be more sensitive than microscopic enumeration. Alternatively, the probe may be hybridizing to organisms other than zebra mussels, despite specificity evaluations conducted during the probe design process. Continued research is required to further evaluate probe specificity and to develop simple probe-based field methods for routine use.

SUMMARY OF KEY CONCLUSIONS

1. Sufficient divergence in the 18S rRNA gene of the zebra mussel exists such that species-specific probes can be developed. A zebra mussel specific probe has been developed.

2. Methods for the detection of rRNA from veligers have been developed. Detection of a single veliger has been demonstrated.

3. Detection of veligers in natural waters by probe technology is generally consistent with microscopic methods. Veliger density may not be directly quantifiable from hybridization strength.

4. Further work is required to confirm the specificity of the probe in field studies.

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