Hydrobiologia **487:** 33–44, 2002.

Specific amplification of the 18S rRNA gene as a method to detect zebra mussel (*Dreissena polymorpha*) larvae in plankton samples

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Received 24 April 2001; in revised form 22 July 2002; accepted 20 August 2002

Key words: zebra mussel, bivalve, veliger, 18S rRNA, PCR

Abstract

An important issue in the management of zebra mussel (*Dreissena polymorpha*) populations is early, rapid, and accurate detection of the planktonic larvae (veliger) of the zebra mussel. The goal of this study was to explore the feasibility of developing a molecular approach for the detection of zebra mussel larvae in diverse environments. In this study a *Dreissena polymorpha*-specific 18S ribosomal RNA gene targeted oligonucleotide primer (ZEB-715a) and Polymerase Chain Reaction (PCR) assay was developed and compared with cross-polarized microscopy as a means to detect zebra mussel veligers in plankton samples. The design of the zebra mussel-specific primer was facilitated by sequencing nearly the complete 18S rRNA gene from the zebra mussel and three other closely related freshwater Veneroids including the quagga mussel (*D. bugensis*), the dark false mussel (*Mytilopsis leucophaeata*), and the Asian freshwater clam (*Corbicula fluminea*). The specificity of the primer for the zebra mussel was empirically tested by using the primer as a direct probe in a blot hybridization format. A single veliger in a plankton sample could be detected by PCR using this approach. Veliger detection sensitivity using the PCR approach was estimated to be over 300 times more sensitive than cross-polarized light microscopy based techniques. Cross-polarized light microscopy and the PCR technique were used to identify the presence of zebra mussel larvae in plankton samples that were collected from a variety of natural and industrial water sources. Detection results (presence or absence) were generally consistent between the two methods. Although additional studies will be required before routine application of molecular based veliger detection technology is available, a long-term goal of this work is the application of molecular 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). Colonization of North American freshwater systems by the zebra mussel has impacted the operation of industrial facilities, interfered with recreational resources, and been held responsible for disrupting ecosystem balances (Ludyanskiy, 1993; Caraco et al., 1994; Khalanski, 1997). Native to the drainage basins of the Black, Caspian and Aral Seas, the zebra mussel was introduced to Europe by at least the mid 1700s (Archambault-Guezou, 1976). Zebra mussel larvae (veligers) were presumably transported from Europe to North America in the ballast water of trans-Atlantic freighters and introduced into the Great Lakes in 1985

or 1986 (Roberts, 1990; Carlton, 1993; Ludyanskiy, 1993).

Since its first introduction to North America, *Dreissena polymorpha* has rapidly colonized U.S. and Canadian fresh water bodies. The colonization success of the zebra mussel in North America is largely related to its method of reproduction and life history. The zebra mussel is unique among freshwater bivalves, except perhaps *Mytilopsis*, because it is a broadcast spawner with external fertilization that produces free-swimming planktonic veligers that are easily dispersed by flowing water. The veliger is a primary means by which zebra mussels colonize new environments, and the presence of zebra mussel veligers can be indicative of successful reproduction (Johnson & Carlton, 1996; Charlebois & Miller, 1998). Therefore, the detection of veligers in the water before settlement occurs is a critical component of current strategies that seek to minimize the risk and/or impact of establishment of mussel populations and protect pristine environments. A number of specific strategies for the management of zebra mussel populations currently target the veliger life stage (Marsden, 1995; O'Neill, 1996; Claudi, 1999). For example, the Wisconsin Electric Power Company (WEPCO) thermally or chemically treats many of its plants that are colonized by zebra mussels only when mussel densities reach critical levels or when spawning occurs and veligers are detected. Other commonly employed management strategies include education and outreach programs (Cassell, 1997), and physical removal of mussels from structures including locks, dams, industrial plants, boat hulls and navigational aids.

Because of their small size (ca. 70–200 microns) and paucity of distinguishing morphological features, identification of bivalve veligers by standard microscopic methods is a tedious and labor intensive process. Therefore, in this study we have investigated the possibility of utilizing molecular genetic approaches to identify and quantify zebra mussel veligers that will ultimately lead to an alternative detection technology and simplify the process of detecting zebra mussels in natural samples. Previous studies have demonstrated the feasibility of developing species-specific oligonucleotide probes and PCR primers that target the 18S rRNA gene of other bivalve species including the bay scallop (*Argopecten irradians*) and the surfclam (*Spisula solidissima*) (Bell & Grassle, 1998; Frischer et al., 2000). In a recent review, Garland & Zimmer (2002) summarize recent advances in the use of molecular techniques for the detection and identification of planktonic larvae. Additionally, a number of other genetic-based population studies have examined the use of genetic markers other than ribosomal RNA genes that could potentially be used as targets for larval detection (Baldwin et al., 1996; Claxton and Boulding, 1998; Claxton et al., 1998; Stepien et al., 1999; Stepien et al., 2001). The goal of the present work was to design a *Dreissena polymorpha*-specific 18S rRNA gene targeted PCR primer and evaluate its use for detecting zebra mussel veligers in samples collected from a variety of natural and industrial environments.

Methods

Development of a Dreissena-specific primer

Animal collection

Adult specimens of *Dreissena polymorpha*, *Dreissena bugensis*, *Corbicula fluminea*, and *Mytilopsis leucophaeata*, were collected for sequencing studies. *Dreissena polymorpha* was collected from several locations in New York State including the Hudson River, Seneca Lake, Canandaigua Lake, and Cayuga Lake. In addition, *D. polymorpha* specimens were collected from Lake Champlain in Vermont. *D. bugensis* was collected from the St. Lawrence River, Lake Erie, and Oneida Lake, all in New York State. *Corbicula fluminea* was collected from Santee Cooper Lake in South Carolina, and *Mytilopsis leucophaeata* was collected from the Mississippi River. Animals were collected and transported to the lab live. All samples were stored at $-80\,^{\circ}$C until use.

Plankton samples

Plankton samples were collected from Lake George water by filtering water through a 44 μm (Ernest Case Co., Andover, NJ) or a 63 μm mesh size (Wildco, Madison, WI) plankton net. Water was pumped through the plankton net using a diaphragm bilge pump (Model 34600-series, ITT Jabsco, Costa Mesa, CA) powered by a 12 volt deep cycle marine battery and equipped with a flow meter. Plankton samples collected from industrial, commercial and public water user facilities were passed through the plankton net by hand with surface water collected using a bucket. Plankton samples were obtained from four different types of facilities and Lake Erie. Water facilities included drinking water supply sources managed by the Columbus Division of Water (Columbus,

OH), fish hatchery waters (Ohio State University, OH), intake water supplies at the Wisconsin Electric Power Company (WEPCO) Oak Creek coal-fired power plant (in Milwaukee, WI), and from the water supply servicing a P.H. Glatfelter Company paper mill (in Neenah, WI). Collectively we refer to these sample types as 'industrial'. Samples were collected by personnel at the individual facilities and sent via express mail on blue ice to our laboratory. All samples were collected during the summer and early fall of 1999. Two sites (Baldridge and Dublin Road) were examined on two separate dates. Replicate plankton samples from approximately 200 l of source water were routinely split into two samples for analysis. One sample was preserved in 25% (final concentration) ethanol, and the second sample was stored frozen at −80 °C. Generally, the ethanol fixed samples were used for microscopic analysis and the frozen samples were used for molecular analysis, although PCR amenable DNA could also be extracted from the ethanol fixed samples.

Isolation of genomic DNA

Genomic DNA was purified from individual animals essentially as previously described (Frischer et al., 2000) except that DNA was isolated from the whole animal rather than muscle tissue only. This procedure routinely yielded from 400 to 800 μg DNA/g tissue of high molecular weight genomic DNA suitable for PCR amplification.

Genomic DNA was extracted from plankton samples using a similar extraction procedure as was used for tissue samples after concentrating the plankton samples. Net concentrated plankton samples (25–50 ml) were further concentrated by centrifugation (5000 × ***g***; 10 min) in 50 cc tubes, transferred to a sterile 1.5 ml microfuge tube and finally concentrated to 50–150 μl by centrifugation in a microfuge at 14 000 × ***g*** for 10 min. The concentration and purity of extracted DNA was estimated spectrophotometrically. Purified template DNA with UV absorbence ratios (A_{260}/A_{280}) between 1.6 and 1.8 were generally most amenable to PCR amplification, regardless of whether the sample originated from tissue or plankton samples.

PCR amplification, sequencing, and phylogenetic reconstruction

Nearly the complete 18S rRNA gene was amplified by PCR from each sample of *D. polymorpha*, *D. bugensis*, *C. fluminea*, and *M. leucophaeata* using the universal 18S rDNA targeted oligonucleotide primers UnivF-15 and UnivR-1765 (Frischer et al., 2000). The sequences of all primers and probes used in this study are shown in Table 1. Primers were synthesized using an ABI DNA/RNA synthesizer (model 394) by CyberSyn (Lenni, PA) or by the Molecular Genetics Facility at the University of Georgia. Each 50 μl PCR reaction mixture contained 1–20 ng of purified bivalve DNA, 40 nmoles of a dNPT mixture (Nucleix Plus, Amersham Life Sciences, NJ), 15 pmol each of primers UnivF-15 and UnivR-1765, 1 μg T4 Gene 32 Protein (Boehringer Mannheim, Germany), 1.5 units of Taq DNA polymerase, 1.5 units of Taq Extender, and 5 μl of 10x Taq Extender buffer. All Taq products were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Each 50 μl PCR reaction was adjusted to 1.5 mM $MgCl_2$ by the addition of 3 μl of a 25 mM $MgCl_2$ solution. Amplification was accomplished using a Techne Genius Thermocycler (Irvine, CA) with 32 amplification cycles [95 °C (45 s), 55 °C (45 s), 72 °C (2.5 min)] initiated after a 3.5 min denaturation step at 95 °C and followed by a 10 min final extension step at 72 °C.

To facilitate sequencing of the 18S rRNA gene from *D. polymorpha*, *D. bugensis*, *C. fluminea*, and *M. leucophaeata*, the 18S rDNA PCR product was cloned into the bacterial plasmid sequencing vector pCR®2.1-TOPO using the TOPO-TA cloning system following the instructions provided by the manufacturer (Invitrogen, Carlsbad, CA). Sequences were determined by automated sequencing at the University of Maine Sequencing Facility (Orono, ME) using an ABI model 373A Stretch automated DNA sequencer. Sequencing reactions were facilitated using the ABI Big Dye® prism dideoxy sequencing dye terminator kit following all manufacturer protocols. Sequence assembly was facilitated using ABI analysis software version 3.3 (ABI-Foster City, CA). A total of three sequencing primers were utilized such that the complete gene sequence was determined in the forward direction using primers M13-F, 3-F, and M13-R (Table 1). The sequences of *D. polymorpha*, *D. bugensis*, *C. fluminea*, and *M. leucophaeata* were submitted to GenBank. The accession numbers are AF305702, AF305703, AF305704, AF305705, respectively. The *D. polymorpha* sequence reported is a consensus sequence derived from three individual mussels collected from Lake Champlain, the Hudson River, and Cayuga Lake in New York State.

Phylogenetic analysis

Sequences were initially aligned relative to previously aligned 18S bivalve rRNA genes available publicly

Table 1. 18S rRNA gene targeted PCR primers used in this study

Primer name	Sequence	Application
UnivF-15	5′-CTG CCA GTA GTC ATA TGC	Universal Primer (forward)
UnivR-1765	5′-ACC TTG TTA CGA CTT TAC	Universal Primer (reverse)
Zeb-715a	5′-AGG GGA CGG TGA CGC	Zebra Mussel Specific Primer
M13-F	5′-TGA CCG GCA GCA	Sequencing primer
3-F	5′-GTG CCA GCA GCC GCG G	Sequencing primer
M13-R	5′-AAC AGC TAT GAC CAT G	Sequencing primer

All primers except Zeb-715a were previously published (Frischer et al., 2000).

through the web in the Ribosomal RNA Database Project (RDP) database (http://rdp.cme.msu.edu/html/ Maidak et al., 2000) using the Clustal W version 1.7 multiple sequence alignment algorithm (Thompson et al., 1994). Alignments were viewed and edited based on secondary structure considerations (Relman et al., 1996) using the Genetic Database Editor (GDE; Smith et al., 1992). Phylogenetic trees were inferred and drawn using the *Treecon* for Windows software package version 1.3b (Van de Peer & De Wachter, 1994, 1997) using the Kimura two parameter model for inferring evolutionary distance (Jin & Nei, 1990). Bootstrap estimates (100 replicates) of confidence intervals were also made using the algorithms in *Treecon*.

Primer design

Sequence strings of 15–25 bp unique to *Dreissena* were identified using the *Find Variable Regions* algorithm available in GDE loaded with the aligned 18S rRNA bivalve database. This database contained 36 full length ribosomal sequences from bivalves. Alignment gaps were retained during identification of probe target locations. Optimal probe target sites were initially identified based on the criteria of exhibiting a minimum of 10% sequence difference between the target species (*D. polymorpha*) and its closest sequenced relative *M. leucophaeata*. Following the initial identification of suitable primer target sites, primers were designed to minimize self-complementarity using the *Predict Secondary Structure* algorithm in the DNAsis software package (Hitachi Software Engineering Co., Tokyo). In general, oligonucleotides with negative Gibbs free energy (Δ g) values were excluded from further consideration. Each oligonucleotide sequence that was identified was checked for uniqueness against other available 18S rDNA sequences in GenBank and the Ribosomal Database Project (http://rdp.cme.msu.edu/html/).

Following primer design based on sequence information, the specificity of primers was determined experimentally in a dot blot membrane hybridization format. Oligonucleotides were labeled with digoxigenin-dUTP using the dig 3′ oligo-tailing system (Boehringer Mannheim Biochemical, Mannheim, Germany) following the manufacturer's instructions and hybridized to heat denatured PCR amplified 18S rDNA from *D. polymorpha*, *C. fluminea*, and *M. leucophaeata*. The 18S rDNA amplified product was denatured at 95 °C for 10 min, chilled on ice, and immobilized onto a charged nylon membrane (Zeta Probe, catalog no. 162-0165; Bio Rad, CA) using a dot blot apparatus (Schleicher & Schuell, Keene, NH). DNA was fixed on the membrane by baking at 80 °C *in vacuo* for 2 h. Initially, pre-hybridization and hybridization were conducted as described by the manufacturer at the calculated hybridization temperature. Initial hybridization temperatures were calculated according to Thein & Wallace (1986). Probe hybridization was detected colorimetrically using the Genius System Six non-radioactive labeling and detection system (Boehringer Mannheim Biochemical, Mannheim, Germany).

PCR detection sensitivity

The detection sensitivity of zebra mussel veligers in plankton samples by PCR using the zebra mussel-specific primer set (UnivF-15 and Zeb-715a) was determined in controlled studies. Known numbers of zebra mussel veligers (up to 25) were added to plankton samples (25 ml) from Lake George that did not initially contain zebra mussel larvae. Prior to conducting this study there had been no reports of zebra mussels in Lake George and earlier microscopic and molecular examination of plankton samples from Lake George demonstrated that zebra mussel veligers were not present (pers. obs.). Following DNA extraction and purification, the presence of zebra mussel larvae

Table 2. Veliger enumeration by cross-polarized light microscopy and PCR in industrial samples

Collection date	[1]Location	[2]Veligers/ 20 ml	Univ PCR	Zebra mussel specific PCR
6/07/1999	Gibraltar Island	954	Yes	Yes
6/07/1999	Hebron	0	Yes	No
7/29/1999	Senecaville	1	Yes	No
8/23/1999	PHG	71	Yes	Yes
8/30/1999	Baldridge	0	Yes	No
8/30/1999	Dublin Rd.	38	Yes	Yes
8/30/1999	Hoover Dam	0	Yes	No
9/09/1999	Baldridge	1	Yes	Yes
9/09/1999	OCPP	182	Yes	Yes
9/21/1999	Dublin Rd.	44	Yes	Yes
9/21/1999	HAP	0	Yes	No

[1] Location of sampling sites: Baldridge, Hoover Reservoir at Sunbury Road Bridge, Columbus, OH; Dublin Rd., Dublin Road Water Plant Intake on the Scioto River, Columbus, OH; Gibraltar Island, Lake Erie at Gibraltar Island, OH; HAP, Hap Cremean Water Plant Intake on the Big Walnut Creek, Columbus, OH; Hebron, Ohio State Fish Hatchery, Hebron, OH; Hoover Dam, Hoover Dam, Columbus, OH; OCPP, Oak Creek Power Plant water Intake on Lake Michigan south of Milwaukee, WI; PHG, P.H. Gladfelter Paper Mill, Lake Winnebago at the Fox River, WI; Senecaville, Ohio State fish hatchery, Senecaville, OH;
[2]Total number of veligers counted in 4 five-ml aliquots of plankton sample.

in each sample was determined by PCR. To ensure that DNA extracts were amenable to PCR amplification, only samples in which 18S rDNA PCR product was produced using the universal 18S rDNA primer set (UnivF-15 & UnivR-1765) were amplified with the zebra mussel-specific primer set.

PCR detection of zebra mussel veligers in industrial water sources

Analysis of plankton samples

A total of 11 samples were examined by both PCR and cross-polarized light microscopy. Samples from each type of industrial water facility were compared. The number of veligers per plankton sample ranged from 0 to 954 in this sample set (Table 2). High quality DNA was extracted and purified from these plankton samples using the DNA purification method for adult bivalves previously developed in our laboratory that was modified by the addition of a RNase digestion (Frischer et al., 2000). Five microliters of extracted DNA, equivalent to approximately 5 l of the original water sample, was used per PCR amplification reaction. Amplification was accomplished using a Perkin Elmer 2400 thermal cycler (Perkin Elmer Corp.) with 30 amplification cycles. Amplification cycling conditions were 94 °C (15 s), 53 °C (15 s), 72 °C (30 s). Amplification was initiated after 3 min at 94 °C followed by a 10-min final extension at 72 °C. Reaction mixtures contained 12.5 μl Qiagen PCR Master Mix (Qiagen, Valencia, CA), 0.3–0.65 μl each of appropriate forward and reverse primers (stock solution 100 ng/μl), 0.5 μg T4 Gene32 protein (Boehringer Mannheim, Germany), 1–5 μl DNA template, and filled to 25 μl with nuclease-free distilled water. In all cases each sample was amplified with both universal and zebra mussel-specific primer sets. Negative results using the zebra mussel-specific primer set (UnivF-15 & Zeb-715a) were disregarded if amplification was not possible with the universal primer set (UnivF-15 & UnivR-1765) indicating insufficient quality of DNA template for amplification. At least triplicate independent extractions and PCR amplification attempts were made to confirm negative reactions.

Detection of zebra mussel veligers by cross-polarized light microscopy

Zebra mussel veligers in plankton samples were enumerated by cross-polarized light microscopy as described by Johnson (1995). Microscopy was facilitated using a Nikon SMZ-2T dissecting microscope equipped with two polarizing filters positioned over the light source and the objective so that the sample was sandwiched between the two filters. The concentration of veligers in each ethanol-fixed plankton sample was determined by counting veligers in four, 5-ml replicate sub-samples. The total concentration of veligers was calculated as described by Brady et al. (1993), accounting for sample dilution due to the addition of ethanol.

Results

Molecular phylogeny

Molecular phylogenetic reconstruction of the evolutionary relationship between *D. polymorpha*, *D. bugensis*, *C. fluminea*, and *M. leucophaeata* based on the comparison of 18S rRNA gene sequences, places each of these species within the order Veneroida with *D. polymorpha*, *D. bugensis*, and *M. leucophaeata* forming a highly supported clade (bootstrap values 100%)

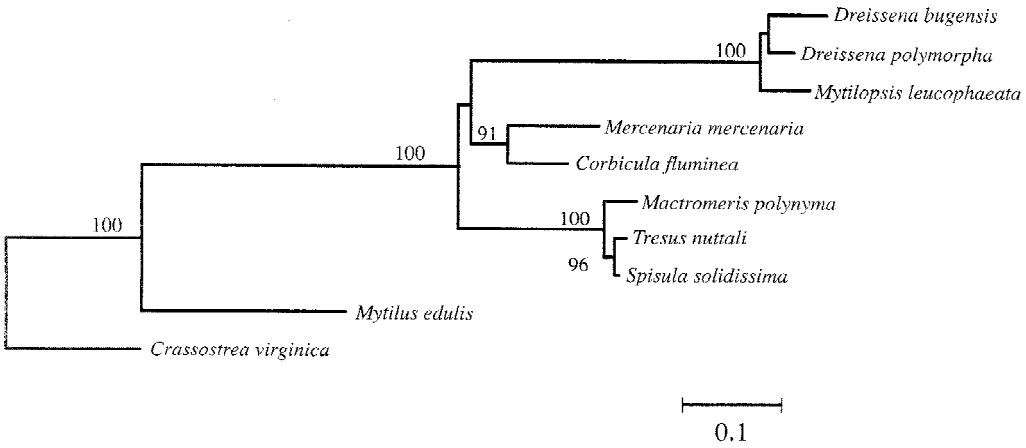

Figure 1. Inferred taxonomic relationship between representatives of the order Veneroida: *Dreissenacea*, *Corbiculacea*, *Mactracea*, and *Mercenaria mercenaria* based on 18S ribosomal RNA gene sequences. The tree was artificially rooted with the 18S rRNA gene from the Eastern oyster *(Crassostrea virginica)* retrieved from GenBank (L78851). The scale bar indicates 0.1 fixed nucleotide substitutions per site. Numbers refer to bootstrap values (from 100) for each node. Bootstrap values below 75 are not shown.

with the closest common ancestor as *Corbicula* (Fig. 1). These results are consistent with the morphologically derived evolutionary history of these species (Rosenberg & Ludyanskiy, 1994).

Probe design

Despite the overall high similarity of the 18S rRNA gene sequence within the Dreissenid phylogenetic cluster, it was possible to identify short sequence stretches that were sufficiently unique from *D. polymorpha*, and *D. bugensis* to serve as a target site for a zebra mussel-specific PCR oligonucleotide primer. Figure 2 shows the target region for the probe designated as Zeb-715a. Over this 16-bp region, the target sequence exhibits a one nucleotide difference from *D. bugensis* and *M. leucophaeata* and two nucleotide differences with *C. fluminea*. This oligonucleotide sequence was unique compared to available 18S rDNA sequences in the GenBank and Ribosomal Database Project databases. As predicted by sequence comparisons, when the oligonucleotide Zeb-715a was labeled with digoxigenin, this probe hybridized only to amplified 18S rDNA from *D. polymorpha* (Fig. 3). Slight hybridization of Zeb-715a with amplified 18S rDNA from *M. leucophaeata* was observed at the calculated theoretical hybridization temperature (47 °C), but this non-specific hybridization signal was removed when pre-hybridization and hybridization temperatures were raised to 53 °C (Fig. 3C). In concurrent hybridization studies, a universally targeted 18S rRNA probe (UnivR-1765) hybridized to replicate blots, indicating that sufficient DNA was placed on the blots for hybridization detection (Fig. 3A). Amplified DNA from *D. bugensis* was not available for these studies. These studies demonstrated the hybridization specificity of the oligonucleotide Zeb-715a for the zebra mussel and confirms that it can be used to distinguish *Dreissena polymorpha* from *Mytilopsis* and other Veneroids.

Probe sensitivity

The sensitivity of the primer Zeb-715a to detect zebra mussel veligers in plankton samples was determined by amplifying zebra mussel-specific 18S rDNA from plankton samples with known numbers of zebra mussel veligers (Fig. 4). In these studies specific zebra mussel PCR product was detected from natural plankton samples collected from Lake George, NY that had been spiked with as little as a single zebra mussel veliger. These studies demonstrate that a detection limit of a single veliger in a 50 ml plankton sample is attainable by PCR (Fig. 4B, lane 4). Based on these studies, it was observed that DNA from a single veliger could be detected in a small fraction (5 l) of a 200 l water sample by PCR. Therefore, detection sensitivity of the PCR technique was estimated to be a single veliger in 67 m^3 of water. This is over 300 times more sensitive than detection sensitivities reported for the cross-polarized light microscopy technique (5 veligers/m^3; Kraft & Johnson, 2000). Specific zebra mussel amplification products were not amplified from Lake George plankton samples that were not spiked with zebra mussel veligers (Fig. 4B, lane 3). Inter-

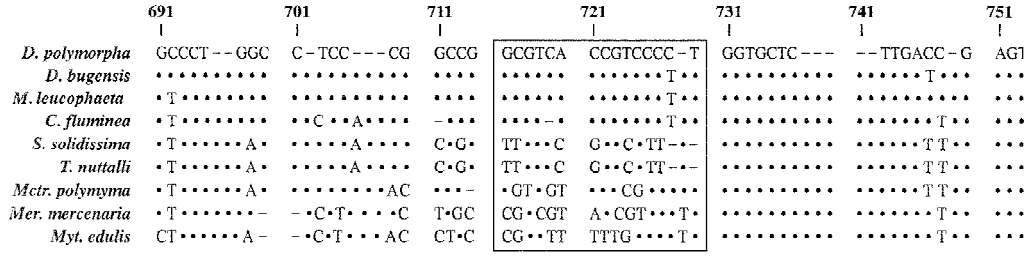

Figure 2. Target region (boxed) in the 18S rRNA gene for the zebra mussel-specific probe Zeb-715a. Aligned sequences from organisms used to develop the probe are shown. Dots (·) indicate regions of exact nucleotide matches and dashes (–) indicate alignment gaps.

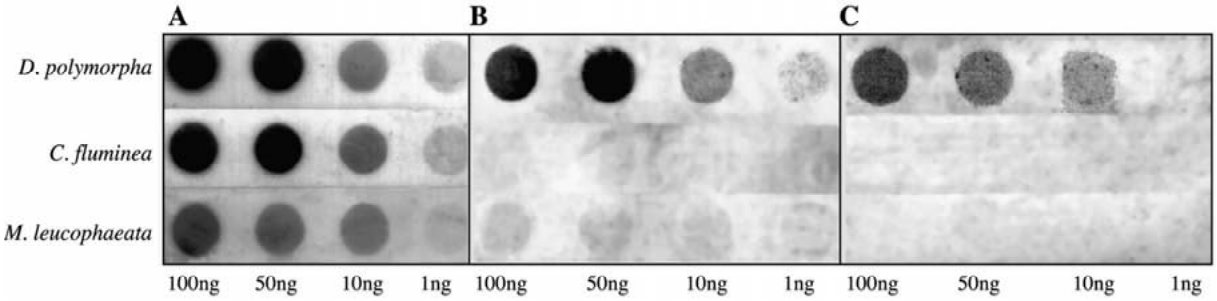

Figure 3. Hybridization and colorometric detection of digoxigenin-labeled. (A) Universal eukaryotic probe UnivR-1765, (B) Zebra mussel-specific probe Zeb-715a at 47 °C and (C) at 53 °C. Zeb-715a was hybridized to denatured PCR-amplified SSU rRNA from *D. polymorpha*, *C. fluminea*, and *M. leucophaeata*.

estingly, a non-specific amplification product (ca. 600 bp) was also amplified by the primer set UnivF-15 and Zeb-715a in all of the Lake George plankton samples, but this fragment was easily distinguished from the expected 701 bp fragment. Although we do not know the identity of the unspecific product, it is unlikely to be related to the zebra mussel since it did not increase in intensity with the quantitative addition of zebra mussel veligers to Lake George water samples. This fragment was not observed in DNA extracted from zebra mussel tissue or the plankton sample from Lake Champlain that contained 50 zebra mussel veligers (Fig. 4B, lanes 1 and 2, respectively). The universal 18S rDNA targeted primer set (UnivF-15 & UnivR-1765) amplified product from all samples indicating that the quality of template DNA from each sample was sufficient for PCR amplification and that high molecular weight 18S rDNA was available in each sample for amplification (Fig. 4A).

Comparison of veliger detection by PCR and cross-polarized light microscopy

The detection of zebra mussel larvae by the molecular PCR technique was compared with detection by cross-polarized light microscopy. The results of these comparisons are summarized in Table 2. In 10 of the 11 direct comparisons between the PCR and microscopy detection techniques the results, presence or absence of veligers, were consistent with each other. In one sample (Senecaville Fish Hatchery) the presence of veligers was indicated by the microscopy technique, but not by PCR.

Discussion

Based on a combination of sequence comparison and empirical testing, we have developed an 18S rRNA targeted oligonucleotide PCR primer specific for the zebra mussel (*D. polymorpha*) that can be used to identify the presence of zebra mussel larvae in plankton samples. Using the existing database of bivalve 18S rRNA gene sequences available publicly through the Ribosomal Database Project and GenBank (Maidak et al., 2000; Benson et al., 2000) and three additional 18S rRNA gene sequences from the zebra mussel (*D. polymorpha*), the quagga mussel (*D. bugensis*), the dark false mussel (*M. leucophaeata*), and the Asian freshwater clam (*C. fluminea*) determined in this study, it was possible to identify an oligonucleotide target site that could be used to differentiate the zebra mussel from other sequenced

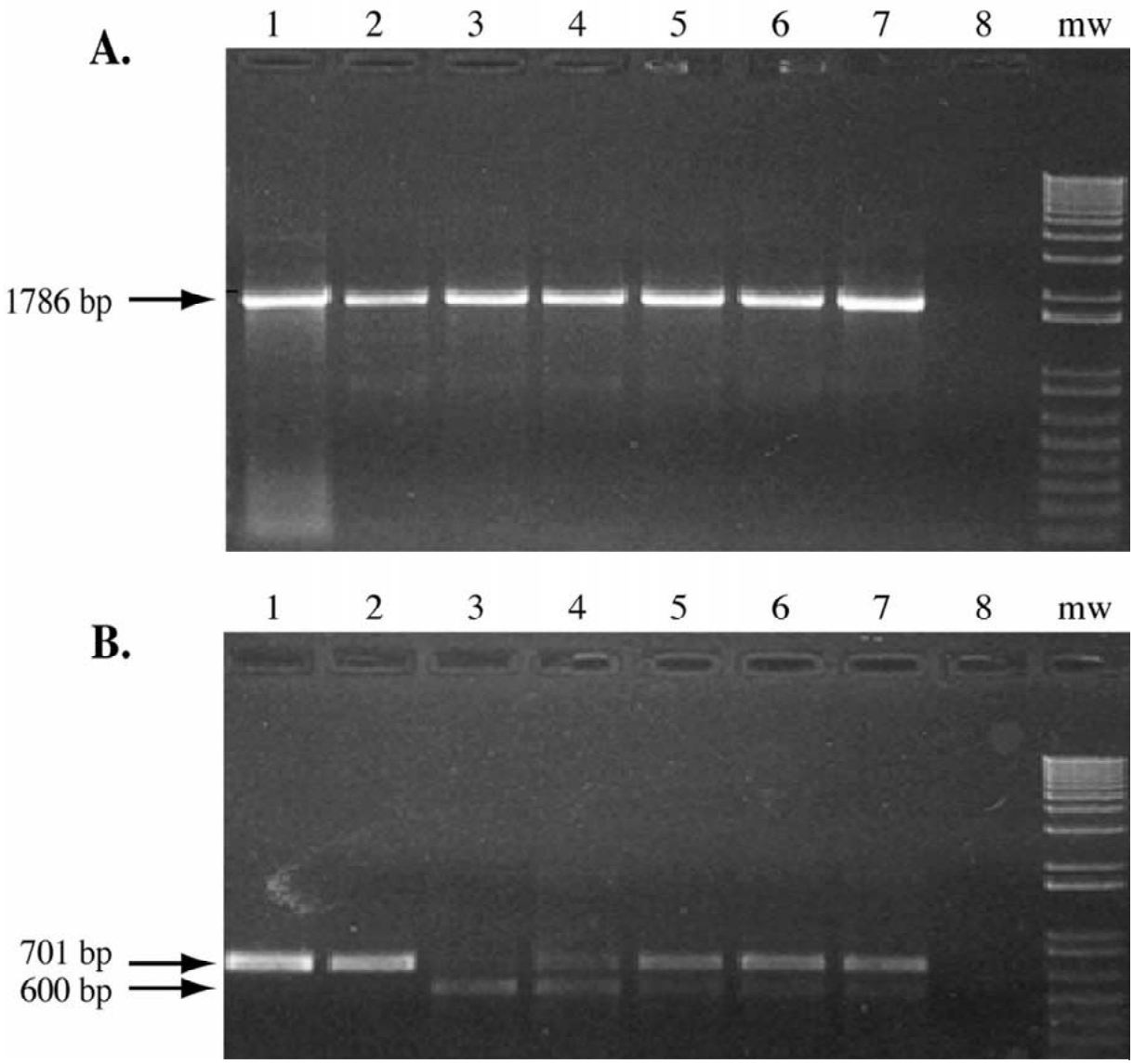

Figure 4. PCR Detection of spiked zebra mussel larvae in plankton sample. (A) PCR amplification of a 1786 bp fragment of the 18S rRNA gene produced using the eukaryotic universal primers UnivF-15 and UnivR-1765 and (B) amplification of the 701 bp zebra mussel-specific amplicon produced using the zebra mussel specific primer set UnivF-15 and Zeb-715a. In both panels (A & B) lane 1, purified zebra mussel DNA (positive control). Lane 2, DNA extracted from a Lake Champlain, VT plankton sample. Lane 3, DNA extracted from a Lake George plankton sample. Lane 4, DNA extracted from a Lake George plankton sample spiked with 1 zebra mussel veliger. Lane 5, DNA extracted from a Lake George plankton sample spiked with 5 zebra mussel veligers. Lane 6, DNA extracted from a Lake George plankton sample spiked with 10 zebra mussel veligers. Lane 7, DNA extracted from a Lake George plankton sample spiked with 25 zebra mussel veligers. Lane 8, negative control (no DNA template). mw, molecular weight marker (1 kb ladder, Gibco BRL, Grand Island, NY).

bivalves based on its 18S rRNA gene sequence. Phylogenetic reconstruction from 18S rRNA gene comparisons were consistent with morphologically derived analyses and placed the Dreissenidae family within the order Veneroida, and suggested a close ancestral relationship with the marine Mactrid clams (Nuttall, 1990; Morton, 1993; Spidle et al., 1994; Rosenberg & Ludyanskiy, 1994; Andrusov, 1898). During these studies we also sampled and sequenced the 18S rRNA gene from zebra mussels collected from several geographic locations. As expected, comparison of these sequences indicated that the 18S rRNA gene was conserved among geographically separate zebra mussel populations (data not shown). These analyses

suggest the validity of the sequence data and provide the appropriate context for the development of species and group-specific 18S rRNA targeted oligonucleotide probes for the zebra mussel. On the basis of sequence comparison, a 16-bp oligonucleotide probe was designed (Zeb-715a) that was specific for the zebra mussel. This probe has at least one unique base pair compared with all other bivalve species available in the current databases and, in most cases, two or more base-pair differences. Compared to its closest relatives (*D. bugensis* and *M. leucophaeata)*, the zebra mussel has a cytosine instead of a thymidine at position 728. Empirical testing demonstrated the specificity of the probe for *D. polymorpha* compared to *M. leucophaeata* and *C. fluminea*. The specificity of the probe for *D. bugensis* was not empirically tested in this study. However, since *D. bugensis* shares the identical sequence of the probe target site with *M. leucophaeata* and *C. fluminea*, it is reasonable to expect that it is possible to differentiate *D. bugensis* from *D. polymorpha* on the basis of this probe. Elsewhere it has been reported that *D. bugensis* and *D. polymorpha* can be separated on the basis of the mitocondrial COI and 16S rRNA gene sequences using a PCR and RFLP approach (Claxton et al., 1998; Claxton & Boulding, 1998; Stepien et al., 1999). Initially, based on sequence comparisons, a total of 18 regions were identified as potential zebra mussel-specific 18S rRNA probe target sites. However, empirical testing of probes targeted to the majority of these sites indicated that they were not suitable as zebra mussel-specific probe targets. Probes targeted to these regions were either not specific for the zebra mussel or would not hybridize to 18S rDNA amplified from zebra mussel tissue. The explanation for these results remains unclear, but it appears to be a common phenomenon since similar results were obtained while designing 18S rRNA targeted probes for other bivalve species including the bay scallop *(Argopecten irradians)*, sea scallop *(Placopecten magellanicus)*, and the hard clam *(Mercenaria mercenaria)*.

Direct comparison between the cross-polarized light microscopy and molecular PCR methods indicated a strong agreement between these methods. Of the 11 samples where a direct comparison could be made, ten were consistent with each other. The 11th sample was derived from a turbid water sample from a fish hatchery pond. In this sample only a single veliger was detected during routine microscopic examination of four 5-ml replicates of a concentrated plankton sample. This result suggests that the PCR detection may be less sensitive than that of the microscopic technique in certain samples. However, assay sensitivity results from this study based on water from an oligotrophic lake (Lake George, NY) indicated that a single veliger in a plankton sample derived from 200 l of water could be detected by PCR using the Zeb-715a primer set and that the PCR technique was over 300 times more sensitive than cross-polarized light microscopy so, had there been veligers in this sample we would have expected to detect them (Fig. 4). Alternatively, since so few veligers were present in this sample (1 per 20 ml plankton sample), the discrepancy between the PCR and microscopy results might have been due to sampling error. It is possible that the subsample examined by PCR did not contain even a single veliger. A third possible explanation for the lack of detection in the Senecaville fish hatchery sample by PCR could be that the microscopic analysis was incorrect. This seems plausible since veligers in this sample were particularly difficult to identify microscopically due to the high density of particles in the samples. Adult zebra mussels were not reported in the Senecaville fish hatchery at the time that the plankton sample was collected (D. Culver, pers. comm.). Finally, a fourth possibility is that the veliger observed was either not a zebra mussel veliger or was dead at the time of collection. Since the PCR technique targets a cellular component of the zebra mussel rather than the shell, only living or recently dead larvae can be detected by the PCR approach.

In earlier studies we reported the use of digoxigenin-labeled 18S rRNA targeted probes for the direct detection and quantification of zebra mussel and bay scallop larvae in plankton samples (Frischer et al., 1997, Frischer et al., 2000). These studies were conducted in samples from a single oligo-mesotrophic lake (Lake George, NY) and Tampa Bay, Florida, respectively. However, in this study a comparison of microscopic identification and direct probe hybridization results derived from a greater diversity of environments suggested that in many of the samples the zebra mussel-specific probe hybridized non-specifically to RNA extracted from plankton samples and immobilized on membrane filters. This non-specific hybridization prevented the use of the probe in a direct format and necessitated the focus on detection by PCR. We speculate that the probe was hybridizing to genes other than ribosomal genes present in plankton DNA extracts, since the probes did not hybridize to PCR amplified 18S rRNA gene fragments from a variety of other closely related organisms. An alternate explanation for non-specific hybridization might have

been a non-specific reaction between the digoxygenin immuno detection system and the extracted plankton sample. It has been reported that non-specific immuno detection can occur when high concentrations of DNA (>400 ng) are present (Boehringer Mannheim, technical service). However, plankton samples that had not been hybridized with the digoxygenin probe did not react with the anti-digoxygenin immuno detection system suggesting that this was not the case. Furthermore, non-specific hybridization in some samples was also observed when probes were directly labeled radioactively with ^{32}[P] indicating that non-specific hybridization was not an artifact of the non-radioactive probe labeling and detection system.

Although in this study it was demonstrated that a molecular approach could be used to reliably detect zebra mussel veligers in a diverse group of water sample types, it has not yet been demonstrated that the PCR technique described in this report will be quantitative or could be used as a routine methodological replacement for microscopy based techniques. Therefore, a direct cost and labor comparison between molecular and microscopy-based detection methodologies would be premature. Ultimately, the long-term objective of this technology is the development of a robust system that can be used to detect specifically bivalve larvae in plankton and/or water samples. Despite the highly conserved nature of the 18S ribosomal RNA gene, the rRNA molecule provides an attractive molecular target for organism-specific probes for a number of reasons. First, because ribosomes are highly abundant in living cells (10 000–100 000 per cell; Alberts et al., 1994), the sensitivity of ribosomally targeted probes is higher than is possible with probes targeted to single or low copy number genes. For example, ribosomal rRNA targeted oligonucleotide probes are routinely used to detect and identify single bacterial cells (Amann et al., 1995; Frischer et al., 1996). Second, because of the relative ease in obtaining rRNA gene sequences, the availability of large databases of ribosomal rRNA genes, and the ease with which large ribosome genetic databases can be aligned, it is possible to design and tailor oligonucleotide probes to a wide variety of organisms including bacteria, fungi, protozoans, and metazoans. In our laboratory, in addition to oligonucleotides specific for the zebra mussel, we have developed or are developing ribosomal rRNA oligonucleotides specific for a number of scallop, clam, mussel, and dinoflagellate species (Frischer et al., 2000; Grubel et al., 2002). Ultimately we envision the availability of probe libraries, perhaps incorporated onto gene chips (microarrays), that could be used to simultaneously assess the presence and abundance of a wide range of organisms in water and plankton samples. However, a larger number of organism-specific probes must first be designed and tested, and a number of technological hurdles must be overcome before this goal can be fully realized and routinely applied in field studies.

Acknowledgements

This work is the result of research funded by the National Oceanic and Atmospheric Administration with award Nos. NA76RG0499 and NA46RG0090 to M.E. Frischer and S.A. Nierzwicki-Bauer through the auspices of the Research Foundation of the State University of New York for New York Sea Grant. The Helen V. Froehlich Foundation also provided partial support of this research. We gratefully acknowledge the cooperation of the Wisconsin Electric Power Company (Milwaukee, WI) and J. Babinec; the Columbus Division of Water (OH) and B. Dorian; P.H. Glatfelter Co. (Neenah, WI), and W. Hartman, Integrated Paper Services (Appleton, WI) and D. Rades; and Dr D. Culver at The Ohio State University. We thank B. Baldwin for providing *D. bugensis* specimens from the St. Lawrence River, Lake Erie, and Oneida Lake. We also wish to acknowledge Kathleen Bass for early technical assistance and Dr Dan Marelli for assistance throughout the project. The U.S. Government is authorized to produce and distribute reprints for governmental purposes notwithstanding any copyright notation that may appear herein. The views expressed herein are those of the authors and do not necessarily reflect the views of the National Oceanic and Atmospheric Administration or any of its sub-agencies.

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