NOTES

A new molecular technique for identifying field collections of zebra mussel (*Dreissena polymorpha*) and quagga mussel (*Dreissena bugensis*) veliger larvae applied to eastern Lake Erie, Lake Ontario, and Lake Simcoe

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Abstract: The veliger larvae of two introduced species of bivalves, the zebra mussel (*Dreissena polymorpha*) and the quagga mussel (*Dreissena bugensis*), are difficult or impossible to tell apart morphologically. We have developed specific dreissenid polymerase chain reaction (PCR) primers from dreissenid bivalve DNA sequences, which amplify a region of the cytochrome *c* oxidase subunit I mitochondrial gene. Non-dreissenid mtDNA, as found in field-collected veliger samples, was not amplified by these new PCR primers. The DNA sequence of this region distinguishes zebra mussel from quagga mussel larvae. Restriction digests of this region using the enzyme *Scr*FI showed no intraspecies variation in restriction pattern. We used this technique to distinguish the species of veliger larvae collected in eastern Lake Erie, Lake Ontario, and Lake Simcoe. In our limited study, no quagga mussel larvae were found in Lake Simcoe, suggesting that this mussel species has not yet spread to the Kawartha Lake system. No zebra mussel larvae were found in either Lake Erie or Lake Ontario. These preliminary results add to the growing evidence that the quagga mussel is replacing the zebra mussel in parts of the lower Great Lakes.

Résumé : Les larves véligères de deux espèces introduites de bivalves, la Moule zébrée (*Dreissena polymorpha*) et la Moule quagga (*Dreissena bugensis*) sont difficiles, voire impossibles, à distinguer par simple examen morphologique. Nous avons créé, à partir de séquences d'ADN de bivalves dreissénidés, des amorces de reactions polymerases en chaîne (RPC) spécifiques aux dreissénidés qui amplifient une région du gène mitochondrial de la sous-unité I du cytochrome *c* oxydase COI. L'ADNmt d'autres organismes trouvés dans des échantillons de larves véligères recueillies en nature n'est pas amplifié par ces nouvelles amorces RPC. La séquence d'ADN de cette région distingue la larve de la Moule zébrée de celle de la Moule quagga. Les digestions de cette région au moyen de l'enzyme de restriction ScrF1 indiquent qu'il n'y a pas de variation intraspécifique du pattern de restriction. Nous avons utilisé cette technique pour distinguer les espèces de larves véligères de l'est du lac Érié, du lac Ontario et du lac Simcoe. D'après cette étude de portée limitée, aucune larve de Moule quagga n'a été trouvée dans le lac Simcoe, ce qui indique que cette espèce n'a pas encore atteint le système hydrographique du lac Kawartha. Aucune Moule zébrée n'a été trouvée dans le lac Érié ou le lac Ontario. Ces résultats préliminaires constituent une autre preuve que la Moule quagga est en train de remplacer progressivement la Moule zébrée dans certaines parties du système inférieur des Grands Lacs.
[Traduit par la Rédaction]

Introduction

In the past decade two species of dreissenid bivalves have been introduced into the Laurentian Great Lakes. The first of these is the zebra mussel, *Dreissena polymorpha* (Pallas, 1771), which was first found in North America in Lake St. Clair in 1988 (Hebert et al. 1989). It quickly spread throughout the Great Lakes and the St. Lawrence River (Griffiths et al. 1991). More recently it has spread to the canals and rivers of eastern North America (Martel et al. 1995). The second dreissenid mussel species was first recorded in North America from Lake Ontario (May and Marsden 1992) and tentatively named the "quagga" mussel. It was subsequently identified as *Dreissena bugensis* (Andrusov 1897) by Rosenberg and Ludyanskiy (1994) and Spidle (1994), who compared its shell morphology with that of museum specimens. The quagga mussel has so far been found in Lake Ontario, Lake Erie, and the St. Lawrence River (Mills et al. 1993). Zebra and quagga mussels show considerable overlap in distribution, and as quagga mussel

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Table 1. Sample sizes, collection sites, dates, and species identification of zebra and quagga mussel veligers.

Collection site	Date	Station No.	Depth (m)	No. of zebra mussel veligers	No. of quagga mussel veligers
Long Point Bay, Lake Erie	Sept. 11, 1995	1	23	0	10
		2	23	0	10
		3	23	0	10
		4	12	0	6
		5	12	0	10
		6	12	0	10
		7	3	0	4
		8	3	0	4
		9	3	0	10
Hamilton Harbour, Lake Ontario	Oct. 11, 1995	1	Surface	0	6
Beaverton, Lake Simcoe	July 10, 1996	1	Surface	18	0

colonization continues, this overlap is likely to increase. Both zebra and quagga mussels are native to rivers in the Black Sea region (Rosenberg and Ludyanskiy 1994), and are thought to have been introduced to North America by the discharge of ballast water from transoceanic vessels (Hebert et al. 1989; May and Marsden 1992).

The zebra and quagga mussel have similar life histories, which include a planktonic veliger larva. Veliger larvae develop several hours after fertilization of the egg and remain free-swimming for a period of between 1 week (Shevtsova 1968) and 5 weeks (Walz 1975). During this period, dreissenid larvae from different locations and species may mix and be transported over large distances by currents or as the result of surface wind effects and other environmental factors (Smylie 1994). As a result, the species composition of larvae and adults may differ considerably at a given location (Smylie 1994). Clearly, identification of veliger larvae to the species level is important in understanding Great Lakes larval ecology. Discriminating zebra and quagga veliger larvae by using morphological characters is difficult if not impossible, even for experts (Nichols and Black 1994; A. Martel, unpublished data; B.S. Baldwin, unpublished data), therefore a simple, economic genetic technique for separating these species would be invaluable.

In a previous study, Claxton et al. (1997) showed that adult and postlarval zebra and quagga mussels could be identified to the species level using restriction fragment length polymorphisms (RFLPs) of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene. RFLP is an extremely powerful tool for distinguishing between species lacking obvious morphological differences (Avise 1994). Restriction enzyme digestion of mtDNA results in cleavage at known restriction sites specific to each enzyme. Analysis of the restriction fragment patterns can be used to detect restriction-site polymorphisms between species. As a result, it is often possible to use these patterns to distinguish between species.

Using the *NIa*IV restriction enzyme, Baldwin et al. (1996) showed that RFLP analysis could be used to distinguish between *Dreissena polymorpha* and *Dreissena bugensis* veligers that had been cultured in the laboratory and extensively cleaned before analysis. However, in their study, COI mtDNA was amplified using universal polymerase chain reaction (PCR) primers developed by Folmer et al. (1994) that amplify mtDNA from other invertebrates, including crustaceans. In our current study, we were unable to use the technique outlined by Baldwin et al. (1996) to identify field-collected veliger larvae because of contamination by non-dreissenid mtDNA found in field-collected veliger samples. As a result, we developed a new RFLP analysis technique that utilizes mtDNA PCR primers, designed from zebra and quagga mussel COI sequences, to amplify mtDNA fragments from individual veligers. These PCR primers were designed to prevent amplification of non-dreissenid DNA. We use this technique to assess the species composition of field-collected veliger larvae from Lake Erie, Lake Ontario, and Lake Simcoe.

Materials and methods

Field sampling of dreissenids

The adult zebra and quagga mussels used in this study were collected on July 12, 1995, from Long Point Bay, Lake Erie, at a depth of 2 m. Samples were placed on ice and immediately transported to the University of Guelph for analysis.

Veliger samples were collected from Long Point Bay, Lake Erie; Hamilton Harbour, Lake Ontario; and Beaverton Marina, Lake Simcoe. Veliger samples collected from Lake Erie were collected on September 11, 1995, from nine sites and three depths in Long Point Bay (Table 1). Sites 1–3 were located approximately 30 km south of the Nanticoke Generating Station, at a depth of 23 m; sites 4–6 were located near sites 1–3, 30 km south of the Nanticoke Generating Station, at a depth of 12 m; and sites 7–9 were located approximately 500 m offshore of the Nanticoke Generating Station, at a depth of 3 m. At each site, water was collected at the appropriate depth using a 5-L Van Dorn bottle. One water sample was collected at each site. Water samples were passed through a plankton net with 53-μm mesh. Veliger samples were rinsed with distilled water into 22-mL plastic vials, then placed immediately on ice for transport to the University of Guelph.

Veliger samples were collected from one depth of Lake Ontario on October 11, 1995, from one site located in Hamilton Harbour at the Canadian Centre for Inland Waters (Table 1). Water samples were collected from the surface using a 20-L bucket. In total, three water samples were collected. Water and veliger samples were treated in the same fashion as those collected in Long Point Bay.

Veliger samples collected from Lake Simcoe were collected on July 10, 1996, from one site located at the Beaverton Marina (Table 1).

Water samples were collected from the surface using a 20-L bucket. In total, three water samples were collected. Water and veliger samples were treated in the same fashion as those collected in Long Point Bay.

Separation of veliger larvae

Samples were viewed and sorted using a stereomicroscope at 32× magnification, using cross-polarized light, as described by Johnson (1995). This method allows for easy recognition of larvae even if foreign debris is present in the samples. Single larvae were removed from the sample and placed in graduated 1.5-mL tubes using a Pasteur pipette. A small amount of sample water was also unavoidably transferred with the veliger larvae. Distilled water was added to each tube after the transfer of the veliger larvae, to bring the total volume in each tube to 100 μL. Only larvae at the "D-shaped" stage were used for this study (Hopkins and Leach 1993).

Isolation of dreissenid mtDNA

Adult mtDNA was isolated as described by Claxton et al. (1997). To extract larval mtDNA, 300 μL of a homogenization buffer solution containing 1.25× SSC (1.87 M sodium chloride and 0.019 M citric acid (trisodium dihydrate)), 1.37% sodium dodecyl sulfate (SDS), and 0.5 mg/mL proteinase K was added to each 1.5-mL tube containing a larva. Samples were then incubated at 55°C for 12 h. The homogenate was then mixed with 130 μL of 5M NaCl and 44 μL of 24:1 methylene chloride : isoamyl alcohol (MCIA), and centrifuged at 2000 × *g* for 5 min, then at 6000 × *g* for 10 min. The supernatant was removed and 400 μL of MCIA added. The mixture was then centrifuged at 6000 × *g* for 5 min. Ethanol precipitation was carried out on the resulting supernatant. The dried pellet was dissolved in 13 μL of sterile distilled water.

Development of dreissenid mtDNA primers and PCR

Initially we used Folmer's universal PCR primers to amplify the COI mitochondrial gene fragment from our veliger samples (Folmer et al. 1994). Restriction digests of the PCR fragment produced a zebra or quagga mussel restriction pattern plus either a 710 base pair (bp) fragment that was resistant to digestion or an unidentified pattern superimposed over the dreissenid restriction pattern. This suggested that the field-collected veliger samples contained non-dreissenid mtDNA, which was amplified by the Folmer PCR primers.

To overcome this problem, we designed PCR primers at regions of the COI sequence that were conserved between zebra and quagga mussels, using sequence data determined by Baldwin et al. (1996). The new primers were used to amplify the COI mitochondrial gene fragment from the veliger samples previously used with the Folmer PCR primers. The new primer pair amplified a 608-bp fragment of the COI mitochondrial gene. The I.U.B. codes of the PCR primer pair (5′ to 3′) used were SCTTGTKGGMACRGGTTTTAGTG (dreissenid A) and GGATCTCCTAACCCTGTWGGATCAA (dreissenid B).

The PCR was performed as described by Innis et al. (1988). We used 1.0 μL of the isolated DNA as the PCR template. The concentration of $MgCl_2$ during the PCR was 4.0 mM. The PCR was carried out for 33 cycles at an annealing temperature of 50°C, the extension was carried out for 45 s at 72°C, and the denaturing step was carried out at 95°C.

Restriction analysis

Restriction analysis of the COI gene fragment of both adults and larvae was carried out using the *Scr*FI restriction enzyme. Restriction enzyme digests consisted of 4.0 μL of PCR product, 4.5 μL of sterile distilled water, 1.0 μL of 10× buffer, and 0.5 μL of *Scr*FI. Digests were incubated at 37.0°C for 18 h, mixed with xylene cyanole at 14%, and then loaded onto 2% agrose gels containing 0.04 mg of ethidium bromide/100 mL. Electrophoresis was carried out for 2 h at 60 mV in standard TBE buffer. Gels were visualized under UV light and photographed.

Results

As in our previous study, restriction analysis of the COI mitochondrial gene of the adult zebra and quagga mussels yielded two distinct restriction patterns. Adult zebra mussels showed three mtDNA fragments of approximately 50, 150, and 400 bp (Fig. 1, lanes 3 and 4), while adult quagga mussels showed four mtDNA fragments of approximately 50, 175, 200, and 250 bp (Fig. 1, lanes 1 and 2).

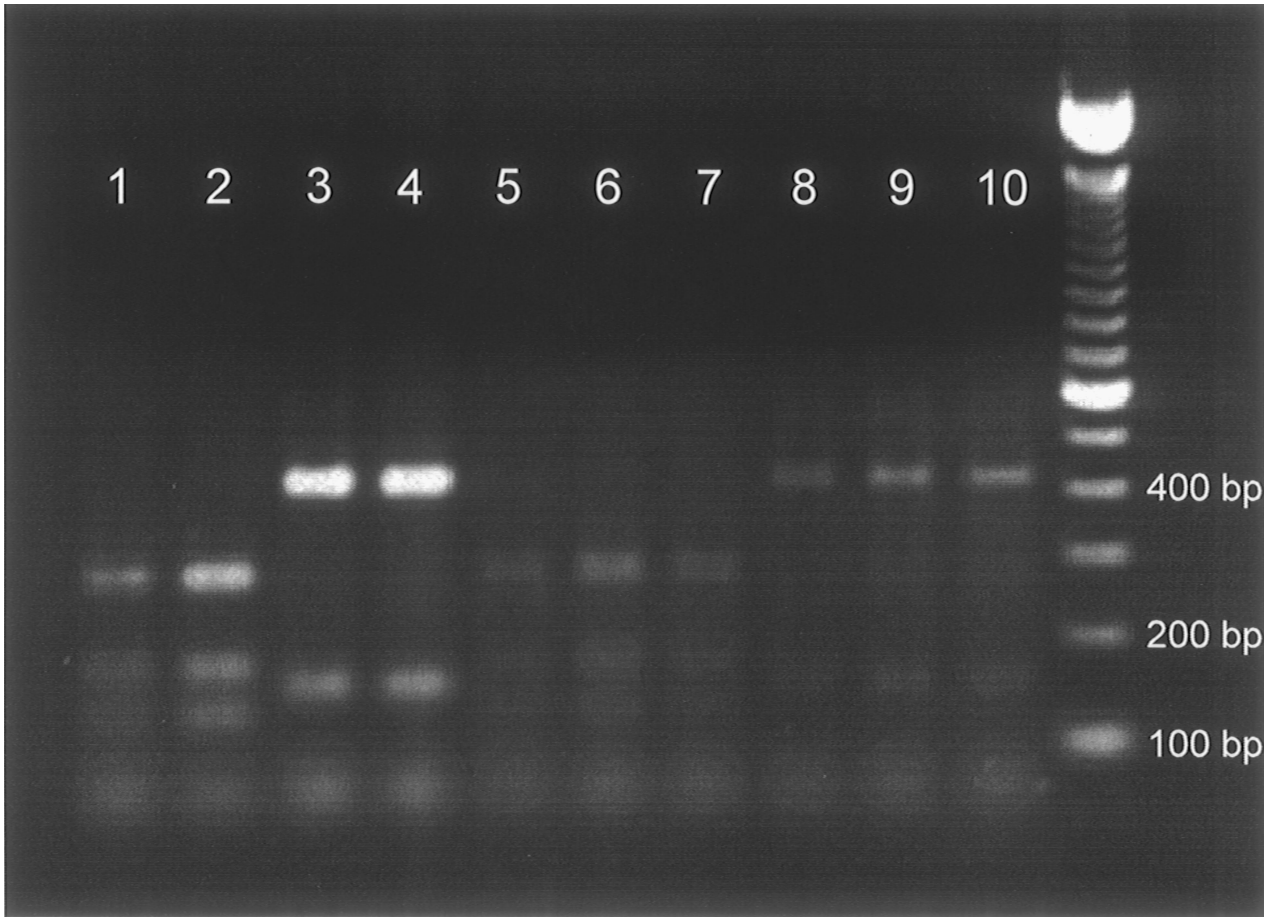
Restriction analysis of the COI mitochondrial gene of the larval dreissenids yielded the same two restriction patterns as we saw in the adults. The pattern shown in lanes 8–10 of Fig. 1 corresponds to *Dreissena polymorpha*, while the pattern shown in lanes 5–7 corresponds to *Dreissena bugensis*. RFLP analysis of veliger larvae collected at all locations yielded one of these two patterns only. No digestion-resistant fragments or alternative restriction patterns were observed for any of the veliger samples (Table 1). All larvae collected at all depths in both Lake Erie and Lake Ontario were quagga mussel veligers, while those collected from Lake Simcoe were all zebra mussel veligers.

Discussion

In this study, we developed PCR primers from two dreissenid mtDNA sequences, which were used to amplify a COI mitochondrial gene fragment of zebra and quagga mussel veligers. However, they did not amplify this region in the non-dreissenid mtDNA known to be present within our field-collected veliger samples, based on previous RFLP analysis using Folmer PCR primers. The plankton samples that contained the zebra and quagga mussel veligers also contained numerous planktonic invertebrates, therefore it is probable that the veliger DNA samples were also contaminated by these invertebrates. RFLP analysis of COI fragment from a mixture of invertebrates is extremely difficult because more than one restriction pattern may be produced. Thus, the specific primers were an improvement on the universal Folmer primers used by Baldwin et al. (1996) for veliger species identification.Owing to the lack of specificity of the Folmer PCR primers, the test veligers used by Baldwin et al. (1996) were cultured under laboratory conditions, and were extensively cleaned before DNA extraction, something which is impossible for field collections.

All of our veliger samples yielded a single restriction pattern, definitive of either zebra or quagga mussel veligers, and no intraspecies variation was noted, which simplified the RFLP analysis. Claxton et al. (1997) also noted no intraspecies variation for either adult or postlarval zebra and quagga mussels. Several studies have considered the possibilty of hybridization between zebra and quagga mussels (Nichols and Black 1993; Spidle et al. 1994, 1995). Spidle et al. (1994) showed a considerable genetic distance between species (Nei's genetic identity, $I = 0.20$), suggesting that hybridization is unlikely. In addition, Spidle et el. (1995) found no natural instances of hybridization in the lower Great Lakes, where the two species co-exist. Our present study is preliminary and was not intended to be a comprehensive investigation of dreissenid veliger species composition in the Great Lakes. However, the lack of zebra mussel veligers in the larval population of Long Point Bay and Hamilton Harbour is worth noting because a well-established population of adult zebra mussels has been

Fig. 1. Restriction digest of a fragment of the COI mitochondrial gene of zebra and quagga mussel adults and larvae. Lanes 1 and 2 represent adult quagga mussels; lanes 3 and 4 represent adult zebra mussels; lanes 5–7 represent quagga mussel veliger larvae; and lanes 8–10 represent zebra mussel veliger larvae.



documented in this area (Mills et al. 1993). Very little research has been done on the relative spawning times and fecundity of *D. polymorpha* and *D. bugensis*. However, environmental conditions such as water temperature and time of year are important factors in triggering zebra mussel spawning (Sprung 1987, 1995). Therefore, it is possible that at the time of collection, environmental conditions in Lake Erie and Ontario may have favoured quagga mussel spawning, leading to elevated numbers of their veligers in the water column.

Several authors have noted a progressive increase in the population of adult quagga mussels relative to adult zebra mussels in both Lake Erie and Lake Ontario (Dermott and Munwar 1993; Mills et al. 1993). The disproportionate number of quagga mussel veligers found in samples collected from the lakes Erie and Ontario may reflect the increasing dominance of quagga mussels in parts of these lakes.

The lack of quagga mussel veligers in Lake Simcoe is not surprising. To date, the range of quagga mussel colonization does not extend beyond Lake Ontario, Lake Erie, and the St. Lawrence River (Mills et al. 1993). Lake Simcoe has only recently been colonized by the zebra mussel (W.T. Claxton, unpublished data).

Both zebra and quagga mussels have colonized large new areas of North America over the past several years (Griffiths et al. 1991; May and Marsden 1992). Continued colonization of new lakes and rivers is likely. Therefore, identification of all life stages of both zebra and quagga mussels, even in areas currently free of dreissenids, is important for aquatic biologists and engineers. Identification of veligers is particularly important, as they pre-date adult colonization.

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An intergeneric hybrid in the family Phocoenidae

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Abstract: A 60-cm female fetus recovered from a Dall's porpoise (*Phocoenoides dalli*) found dead in southern British Columbia was fathered by a harbour porpoise (*Phocoena phocoena*). This is the first report of a hybrid within the family Phocoenidae and one of the first well-documented cases of cetacean hybridization in the wild. In several morphological features, the hybrid was either intermediate between the parental species (e.g., vertebral count) or more similar to the harbour porpoise than to the Dall's porpoise (e.g., colour pattern, relative position of the flipper, dorsal fin height). The fetal colour pattern (with a clear mouth-to-flipper stripe, as is found in the harbour porpoise) is similar to that reported for a fetus recovered from a Dall's porpoise to off California. Hybrid status was confirmed through genetic analysis, with species-specific repetitive DNA sequences of both the harbour and Dall's porpoise being found in the fetus. Atypically pigmented porpoises (usually traveling with and behaving like Dall's porpoises) are regularly observed in the area around southern Vancouver Island. We suggest that these abnormally pigmented animals, as well as the previously noted fetus from California, may also represent hybridization events.

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