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Freshwater mussels (Bivalvia:Unionidae) represent a diverse group of benthic macroinvertebrates that dominate faunal components of streams, rivers and lakes. Unable to avoid the cumulative effects of pollution, pesticides and heavy metals, many species face range reductions and potential extirpation from the state.

In this study a nonlethal DNA isolation technique was developed for use with freshwater mussels. Genetic analysis was performed using Randomly Amplified Polymorphic DNA (RAPD) and dendrogram profiling. Three random primers designated 1050, 1070 and 1283 were used to study genetic diversity between 3 species of mussels from the Neosho and Verdigris Rivers.

All 3 primers detected differences between river drainages and species as well as diversity within species. Band pattern analysis following amplification from 82 mussels using primer 1050 and 1070 generated 11 different sized bands, whereas primer 1283 revealed 17 different sized bands. Dendrogram profiles revealed 24 groupings for primer 1050, 21 groupings for primer 1070, and 24 groupings for primer 1283.

Dendrogram profiles using all 3 primers to test for differences within species and between river drainages using primer 1050 revealed 6 unique biotypes in the Verdigris River and 2 in the Neosho River for *Quadrula quadrula*. Repeating this with primer

1070 revealed 10 unique biotypes in the Neosho River and only 4 unique to the Verdigris River for *Quadrula pustulosa.* Dendrogram profiles using primer 1283 revealed 11 different groupings for *Quadrula pustulosa*, with 7 unique biotypes in the Neosho River and only 1 unique to the Verdigris River, supporting the idea that the Neosho River has more diversity.

A mark and recapture study was also done to confirm survivability following the tissue biopsy. Overall recapture efforts resulted in a 65% total recovery, with 56% coming from the Neosho River and a 78% from the Verdigris River. *Quadrula metanevra* made up the largest percentage of mussels recovered from the Neosho River, constituting 63%, while *Quadrula quadrula* and *Quadrula pustulosa* made up 55% and 45% respectively. Recovery results from the Verdigris River revealed *Quadrula quadrula* had the highest recovery rate with 80%, while *Quadrula pustulosa* made up 71%.

The overall ability of RAPD analysis in this study to discern unique biotypes, combined with the nonlethal tissue biopsy proved to be a successful method for performing genetic analysis on freshwater mussels.

A Nonlethal Genetic Study of Freshwater Mussels

Using Randomly Amplified Polymorphic DNA (RAPD) Analysis

A Thesis Submitted to the

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by

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PREFACE

This thesis was prepared following the publication style of the American Society for Microbiology.

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Introduction

Mussel background

Freshwater mussels (Bivalvia:Unionidae) represent a diverse group of benthic macroinvertebrates that dominate faunal components of streams, rivers and lakes (2,8,13). Serving as natural biological filters, mussels continually sample water chemistry through their incurrent and excurrent siphons. In the process, mussels are exposed to the cumulative effects of toxins such as pesticides, heavy metals and fecal coliform bacteria present in our rivers and lakes (5,9). Unable to avoid the effects of pollution, these long lived invertebrates serve as excellent indicators of water quality (9). Ecologically, mussels are an important link in the food chain of higher vertebrates such as otters, racoons, muskrats, minks, fish and some birds. Found on all but one continent (Antarctica), mussels play a key role in ecology and aquatic diversity (16).

Mussel statistics

There are 297 species of mussels in North America and 1000 species worldwide. Of the 297 North American species, 22 are extinct, 57 threatened and endangered, and 67 are listed as species of concern (10). In Kansas, mussel populations have faced a steady decline for the last century. Three species, *Ligumina recta*, *Epioblasma triquetra* and *Obovaria olivaria* have been extirpated while several other species once common to the state are facing a reduction in their native range (7). Contributing factors to declining mussel populations include building of impoundments, over harvesting, and pollution (4).

Besides destroying a river's natural habitat, impoundments have a profound effect on water quality. In 1981 Kansas Department of Health and Environment (KDHE) conducted a study to determine the effects of impoundments on water quality in Kansas. Initial findings suggested that turbidities were generally lower downstream of impoundments at many sites in Kansas. However, it was discovered that in the Flint Hills where turbidities had traditionally been low, the addition of several major impoundments had raised levels of turbidities considerably. In addition to increased turbidities high levels of organic carbon and chlorophyll were recorded below impoundments in the Flint Hills (16).

Over harvesting can also have a profound effect on mussel populations. Currently in Kansas, 5 species, *Quadrula metanevra* (Monkeyface), *Quadrula quadrula* (Mapleleaf), *Amblema plicata* (Threeridge), *Potamilus purpuratus* (Bluefers) and *Corbicula fluminea* (Asian clam) can be harvested commercially from April 1 to September 30. During the last decade, 1,154.1 tons of mussels have been harvested from Kansas waters. In 1996 mussel harvesting in Kansas reached a peak for the decade with 721,000 lbs harvested in 1 year. Following the 1996 harvest, mussel totals decreased significantly over the next 3 years. In 1998 harvest totals plummeted to 25,100 lbs, a 97% reduction in tonnage in only 2 years. The 1999 harvest showed little improvement with 30,500 lbs harvested. While this reduction in tonnage may in part be explained by market fluctuations and a decrease in permit sales, some portion must be attributed to diminishing populations (9).

Water quality

In 1972, the Clean Water Act was established which called upon the Environmental Protection Agency (EPA) as well as state agencies to develop Total Maximum Daily Loads (TMDL) where water quality was impaired. TMDL is the maximum amount of pollution a stream or lake can receive without violating water quality standards. Water quality standards for developing TMDL are based on designated uses such as recreation, irrigation, groundwater recharge and domestic water supply (5).

In 1992, section 303(d) was added to the Clean Water Act which identified those water bodies which are water quality impaired. All 12 of the major river basins in Kansas have stream segments or lakes listed in the 303(d) list. Some of the main contributing factors to water body impairments are fecal coliform bacteria, pesticides, suspended solids and heavy metals (5). High levels of fecal coliform bacteria as well as pesticides can contribute to a decrease in species richness and abundance. High levels of suspended solids adversely affect the normal rate of oxygen uptake and nitrogen secretion in mussels. Heavy metals are of special concern in the Southeastern part of Kansas due to the mining industry around the Pittsburg area. Even small amounts of heavy metals getting into an aquatic ecosystem can potentially eliminate large numbers of mussels (10). These contributing factors as well as others previously mentioned have resulted in a decline in mussel populations, adding an urgency for government agencies to gather information on population dynamics and biodiversity (4).

Biodiversity

Many experts believe that in order to restore biodiversity it is important to pay particular attention to disturbances in the ecosystem that are slow and less obvious. They advocate protecting ecological processes such as the entire stream ecosystem instead of just trying to preserve 1 or 2 species that we think are in danger and in need of our protection. In other words we should change our philosophy on conservation to one that deals with the ecosystem and its biodiversity as a whole. By protecting and preserving natural ecosystems such as rivers and streams we will increase the ability of species to survive. This survival is dependent on our understanding of how a population's genetic diversity influences the overall biodiversity of the ecosystem. Biodiversity is the fuel that drives the engine of evolution, therefore a loss of biodiversity equates with extinction (15).

Genetic diversity

Understanding the fundamentals of population management in order to restore biodiversity involves evaluating a species genetic diversity (3). Species which exhibit a decline in population numbers exhibit a decrease in heterozygosity which in turn may decrease genetic diversity within a species. Decreased genetic diversity may result in a population bottleneck as well as limiting an organism's ability to adapt to selective forces. Alternately, high genetic diversity allows a population to better adapt to selective pressures and a changing environment. Therefore, in order to implement efficient conservation methods to restore population numbers, biologists must first analyze and

understand a population's genetic structure (4).

Mussel conservation

Typically in the past, techniques for analyzing genetic diversity required large amounts of tissue, subsequently this required killing study specimens (1). Such approaches are foolhardy and impractical when working with endangered species or declining populations. Now however, with advancements in science and because many genetic studies are limited by tissue quantity, quality and availability, many researchers have employed Polymerase Chain Reaction (PCR) techniques which have proven effective for amplifying numerous polymorphic loci from comparatively small amounts of tissue (3).

Polymerase chain reaction

The Polymerase Chain Reaction is a process based on the fact that every cellular organism replicates its own DNA. A typical PCR program cycles approximately 35 times and consists of 3 major phases, denaturing, annealing and primer extension. The denaturing phase is first, usually lasting between 3 and 5 minutes at 94° C. This is followed by a 1 minute annealing phase indicated by a drop in temperature as heat stable polymerase binds to the 3' OH tail of the short (8-25 bases) synthetic polynucleotide annealed to the target DNA. The temperature of the annealing phase is determined by the T_m (the temperature at which half the potential binding sites are thought to be bound) of the primer being used. The specific priming event is initiated by oligonucleotide binding

to its complementary sequence on the template or target DNA. Specifically, oligonucleotide extension of the 3' OH end of the primer. The single stranded template DNA is replicated in a 5'–3' direction as deoxynucleotide triphosphates (dNTPs) are attached to the newly forming double stranded molecule by the DNA polymerase. This 72° C extension phase usually lasts 1 to 2 minutes and ends on most molecules when the enzyme runs off the template strand. Amplification protocols vary in volumes and concentrations based on the method being used. Accordingly, PCR components and parameters should be optimized to increase the efficiency and yield of the reaction while at the same time reducing unwanted products which may result in misleading artifacts (3).

Randomly amplified polymorphic DNA

Randomly Amplified Polymorphic DNA (RAPD) analysis is one of many PCR techniques used to amplify segments of an organism's genome. RAPD typing enables researchers to produce a genetic fingerprint without any prior genetic knowledge of an organism, therefore serving as a powerful tool for studying population genetics and species relatedness in organisms with uncharacterized genomes or the lack of codominant markers (3). Short sequence primers (10-12 bases) are randomly selected and allowed to bind at low annealing temperatures. The results are analyzed using gel electrophoresis to determine which primers produce one or more amplicons (14). Random primer binding generally facilitates amplification of several corresponding sequences of DNA in an organism's genome resulting in several polymorphic loci (11). Because priming sites between organisms vary in proximity, length and location RAPDs are sensitive enough to

detect differences within and between species. The genetic fingerprint can then be analyzed and comparisons made for similarity, relatedness, and diversity based on the number of polymorphic bands. RAPDs have also become popular in genetic studies because they sidestep the time consuming process of primer design as featured in microsatellite analysis. RAPDs can be used to successfully generate population markers linked to phenotypic and genotypic characteristics across taxonomic groups. Microsatellite analysis with the same primer may produce misleading results if used on different species (3). One criticism of RAPD typing has been with reproducibility. However, in this study and many others, it has been shown that optimizing conditions, standardizing reaction volumes and concentrations, as well as repeated use of the same instruments and equipment during the experiment considerably reduce problems with band pattern reproducibility (11). Without these measures band pattern validity is in question, as the distinction between PCR artifacts and amplicons can be difficult. Furthermore, completing a genetic study without killing the organism supports the idea that nonlethal techniques are a viable approach to performing genetic analysis on mussels. Particularly when nonlethal studies produce results as good or better than traditional methods, as was the case in this study.

Materials and Methods

Collection and study area

A total of 85 specimens, 45 *Quadrula quadrula*, 19 *Quadrula pustulosa*, and 21 *Quadrula metanevra,* were collected on 3 different occasions between August 2 - August 22, 1999. Two different sites were selected for collection on the Neosho and Verdigris Rivers in eastern Kansas based on habitat and stream size. The first site was located on the Neosho River, 3.2 km west of Leroy below highway K-57 bridge, Coffey Co., KS. The second site was located on the Verdigris River, 4 km east of Madison below highway 57 bridge, Greenwood Co., KS. Both sites consisted of a shallow riffle with a sand-gravel substrate opening up into a 1-2 meter (m) pool. At each site mussels were collected by hand sampling and identified in the field. All 3 species were collected from both sites with the exception of *Q. metanevra,* which was only found in the Neosho River. The lack of *Q. metanevra* in the Verdigris River at Madison may in part be explained by a decrease in genetic diversity as suggested by the findings in this study.

Mussel storage

After all the specimens were collected they were immediately transported to Emporia State University, Science Hall 60, cold room, where they were housed in twelve 37.8 Liter (L) tanks. Each tank was set up with 8-10 centimeters (cm) of gravel substrate, a Whisper Power Filter, air stone, thermometer and a Penn Plax Therma Flow PC Heater. An Aquaculture double pumping aquarium air pump was used per 2 tanks, and the temperature was kept at 4.4° C. Seven mussels were stored in each tank according to

river drainage. An additional 20 specimens of *Q. quadrula* collected from Reading Lake in Lyon Co., KS were used to develop the nonlethal technique. They were also stored in the cold room except they only had and air supply and substrate and were housed in 2 Rubbermaid 17.8 L dishpans. Each of the 37.8 L tanks received 10 drops of egg laying fry fish food and the filters shut off daily for 12 hours during the first 2 weeks of the experiment. After 2 weeks a majority of the mussels had completely buried themselves in the substrate, therefore feeding was discontinued.

Tissue sample

For each specimen, approximately 30 milligrams (mg) of foot tissue was removed using sterile technique. To do this a 10 millimeter (mm) wide flat head screwdriver was inserted between the valves and gently turned 180° to separate them. Once separated, the round wooden end of a dissection needle was placed between the valves to hold them open and the screwdriver was removed. Small dissection scissors were used to go between the valves and snip approximately a 30 mg piece of foot tissue. The tissue was weighed on a Fisher Scientific Analytical balance and immediately placed in a 1.5 milliliter (mL) tube on ice. A file was then used to make an X in the umbo region of each mussel for mark and recapture. A small bucket of water was used to keep the mussels in until tissue from all specimens had been removed. After tissue was removed, all the mussels were taken back to the cold room and put in their respective tanks.

DNA isolation

Traditional DNA isolation protocols (12) were tested but required large amounts of tissue. A Release-IT DNA Amplification Kit from CPG Incorporated was also tested as a nonlethal approach to studying mussel genetics for several months but demonstrated problems with reproducibility. A Wizard Genomic DNA Purification Kit from Promega was used to isolate the DNA from the 30 mg of foot tissue. Section III D, the Animal Tissue (Mouse Tail) protocol was modified for use with mussel tissue. For all tubes containing 30 mg of tissue to be processed, 120 microliters (µl) of 0.5 Molar (M) Ethylene diamine terta-acetate (EDTA) pH 8.0 was added to 500 µl Nuclei Lysis solution (manufacture's protocol) in separate 1.5 milliliter (mL) tubes and pre-chilled on ice. Six hundred µl of the pre-chilled EDTA/Nuclei Lysis Solution from each tube was then added to the 1.5 ml centrifuge tubes containing the mussel tissue. Next 35 µl of 10 mg/ml Proteinase K (Fisher Scientific) was added to each tube. The tubes were then placed into 140 mL Midwest Scientific vials with each vial containing 2 tubes, arranged one on top of the other. The vials were rotated on high speed (15) in a Hybaid Minihybridization oven at 55° C for 12 to 18 hours or until the tissue was completely digested. Once the samples were digested, 3 µl of RNase (manufacturer supplied) was added to each tube, inverted 25 times and incubated in a 37° C waterbath for 25 minutes. After incubation the tubes were removed and allowed to cool to room temperature for 5 minutes. Next, 200 µl of protein precipitation solution (manufacture's protocol) was added to each tube followed by vortexing at high speed for 20 seconds with a Fisher Vortex Genie 2. The vortexed samples were chilled on ice for 5 minutes before being centrifuged for 4 minutes

in a Beckman Microfuge Lite centrifuge at 13,000 rpm for 4 minutes. At this point the supernatant containing the DNA was poured off of each tube into clean 1.5 ml tubes containing 600 µl of room temperature isopropanol. The tubes were mixed by inversion for 1-2 minutes, then centrifuged for 1 minute at 13,000 rpm. The supernatant was poured off and discarded and the pellet was resuspended and washed by inversion in 600 µl of room temperature 70% ethanol. After washing the pellet the tubes were again centrifuged at 13,000 rpm for 1 minute and the ethanol pipetted off. All tubes were then placed in a Savant Integrated Speed Vac System on medium for 5 minutes to dry the pellet. The dried DNA pellet was resuspended in 100 µl of rehydration solution (manufacture's protocol) overnight at 4^{o} C. Each DNA sample was analyzed at a 260/280 ratio to determine nucleic acid concentration and protein contamination using a Beckman DU 530 Life Science UV/VIS Spectrophotometer.

Polymerase chain reaction

Five hundred nanograms (ng) of DNA was used in 100 µl amplification reactions for each specimen analyzed. Amplification reactions for primers 1050 and 1283 contained final concentrations of 74 µl sterile water, 0.2 millimolar (mM) deoxynucleotide triphosphates (dNTPs), 10x PCR buffer containing 50 mM TrisCl, 50 mM KCL, and 0.01% Triton X-100, 1unit/µl Taq polymerase, 50 micromolar (µM) random primer, and 1.5 mM Magnesium Chloride ($MgCl_2$). Reaction volumes and concentrations stayed the same for primer 1070 except 3 mM $MgCl_2$ was used. Each amplification reaction was performed in an Eppendorf Scientific Gradient Mastercycler

using thin walled PCR tubes and a heated lid. Prior to RAPD analysis, 15 random primers were screened for their ability to differentiate between species. The 3 random primers that were best able to distinguish between species were chosen for use in this study. These primers were optimized for annealing temperature and $MgCl_2$ concentration in a PCR reaction (Fig.1). Optimal amplification conditions for primer 1070 included a 40° C annealing temperature with 3 mM $MgCl_2$. Optimal amplification conditions for primer 1050 and 1283 included a 40° C and 37° C annealing temperature respectively, with 1.5 mM $MgCl_2$. Each primer was then tested in a PCR reaction with each of the 3 species. Results were visualized using a 1.5 % agarose gel (Fig. 2). All 3 primers were able to detect genetic differences between the 3 species based on band pattern analysis. Amplification conditions consisted of an initial step at 94° C for 4 minutes, followed by a 35 cycle repeat. Each cycle included a 1 minute denaturation phase at 94° C; 1 minute annealing phase at either 37° C or 40° C (depending on the primer); 1 minute extension phase at 72° C. An additional second was added to the annealing and extension phase of each cycle for every repeat. The 35 cycles were followed by an additional extension phase of 5 minute at 72°C. Reaction conditions and parameters are listed in Table 1.

Electrophoretic analysis

PCR products were analyzed using agarose gel electrophoresis. A 1.5 % agarose gel was prepared using 100 mL of 1X TAE made from a 50X TAE stock solution (242 g Tris, 57.1 ml glacial acetic acid, 0.5 M EDTA in 1 L of water), 1.5 g of molecular grade agarose (Fisher Biotech) and 6 µl of 10 mg/ml ethidium bromide from Sigma chemical

(No. E-8751). Twenty-eight well gels were ran at 105 volts for 2 hours using a Maxicell EC360M electrophoretic gel system with 15 µl of PCR product plus 5 µl of 10X loading dye (0.25 g bromophenol blue, 50.00 g sucrose, 1 ml of 1 M Tris pH 8 in 100 ml distilled water) in each well. Eight µl of HiLo DNA markers were used in the first and the last lanes. PCR products were visualized and photographed with an ultralum CCD camera and Transilluminator FBTIV-88 from Fisher Biotech, using Scion Image software.

Reproducibility

The last part of the experiment was a repeatability study. DNA was isolated 3 separate times from 2 different mussels. RAPD analysis was performed on all 6 samples and those samples were compared electrophoretically to previous PCR products with the same primer to test each DNA isolation and PCR product for reproducibility and accuracy.

Data analysis

Dendrogram analysis was performed using SPSS 9.0 for windows to statistically analyze the data. Dendrograms were created comparing similarity between all 3 speceis using the 82 DNA samples with each primer. Dendrograms were also created to test for differences within species and river drainage.

Mark and recapture

All mussels were returned to their respective river drainage on January 8, 2000

(representing approximately 5 months in captivity). The mussels were grouped together in a 1x1 m area of the river which was marked by stakes and a flag for their recapture. Before the mussels were placed back in the river careful consideration was given to the possibility of predation and flooding and how these factors might influence recapture results. Sites were chosen a few feet from the main channel in deeper pools of slack water several yards from each shoreline in an attempt to reduce predation and displacement during flooding. The first recapture effort took place on April 25, and the second and final recapture effort took place on May 19, 2000 (approximately 4 months after being returned to the river). Specimens were recorded and tallied before being returned to the river.

Fig. 1. 1.5 % agarose gel of primer 1070 PCR products optimized for annealing temperature and $MgCl_2$ concentration. Magnesium chloride was titrated in 0.5 mM increments. Annealing temperature was optimized using an Eppendorf Gradient Scientific Mastercycler programmed for 1° C temperature increases across the block. Optimal amplification conditions for this primer included a 40° C annealing temperature with 3 mM $MgCl_2$.

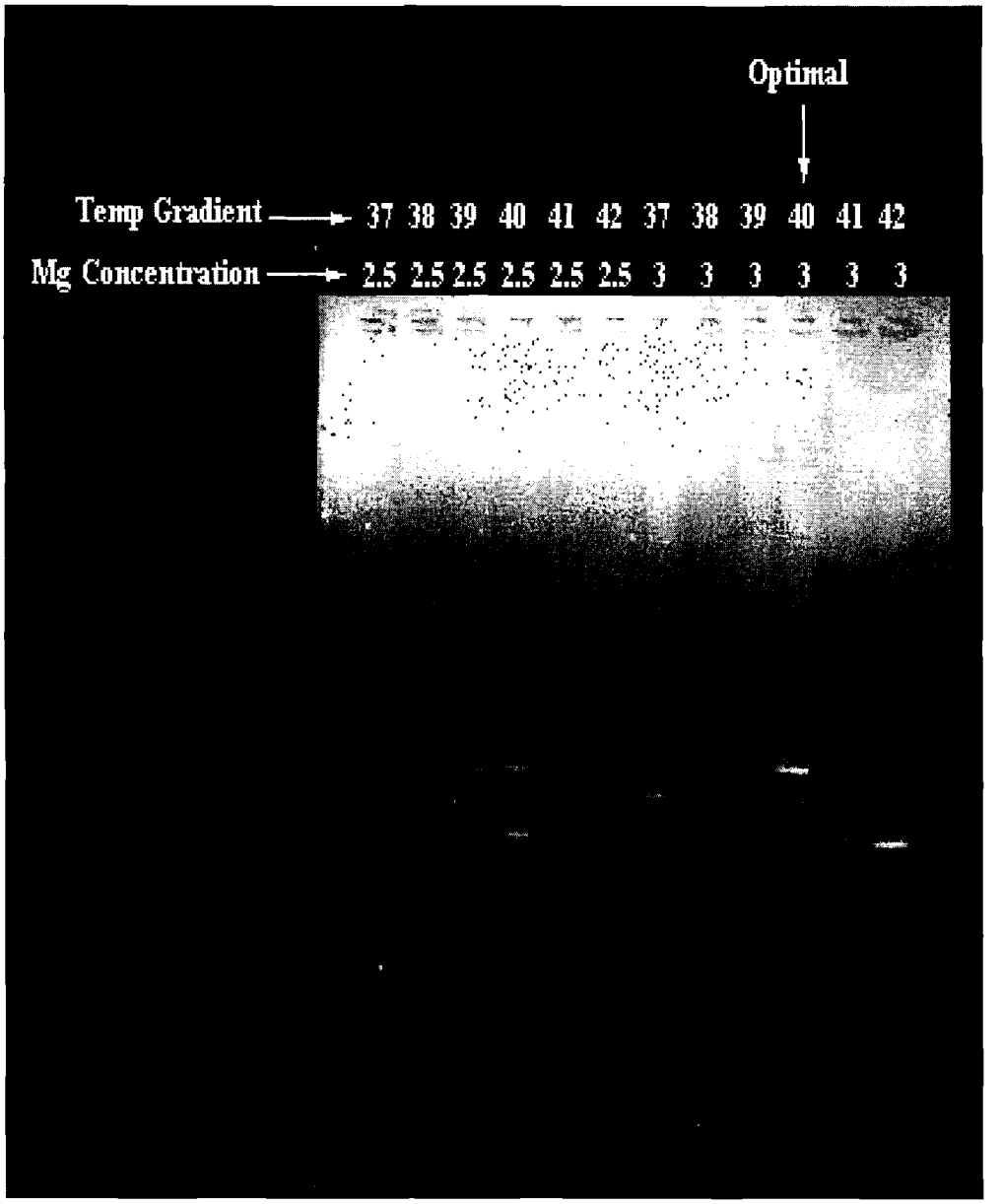

Optimal
Temp Gradient
37 38 39 40 41 42 37 38 39 40 41 42
Mg Concentration
2.5 2.5 2.5 2.5 2.5 2.5 3 3 3 3 3 3

Fig. 2. 1.5 % agarose gel of RAPD primers revealing genetic differences between the 3 species. Each species was abbreviated using its common name. *Quadrula quadrula* (Maple leaf) is Ml, *Quadrula metanevra* (Monkey face) is Mf and *Quadrula pustulosa* (Pimple back) is Pb. Although primer 1254 is featured in this gel, it was not used in this study due to problems with reproducibility.

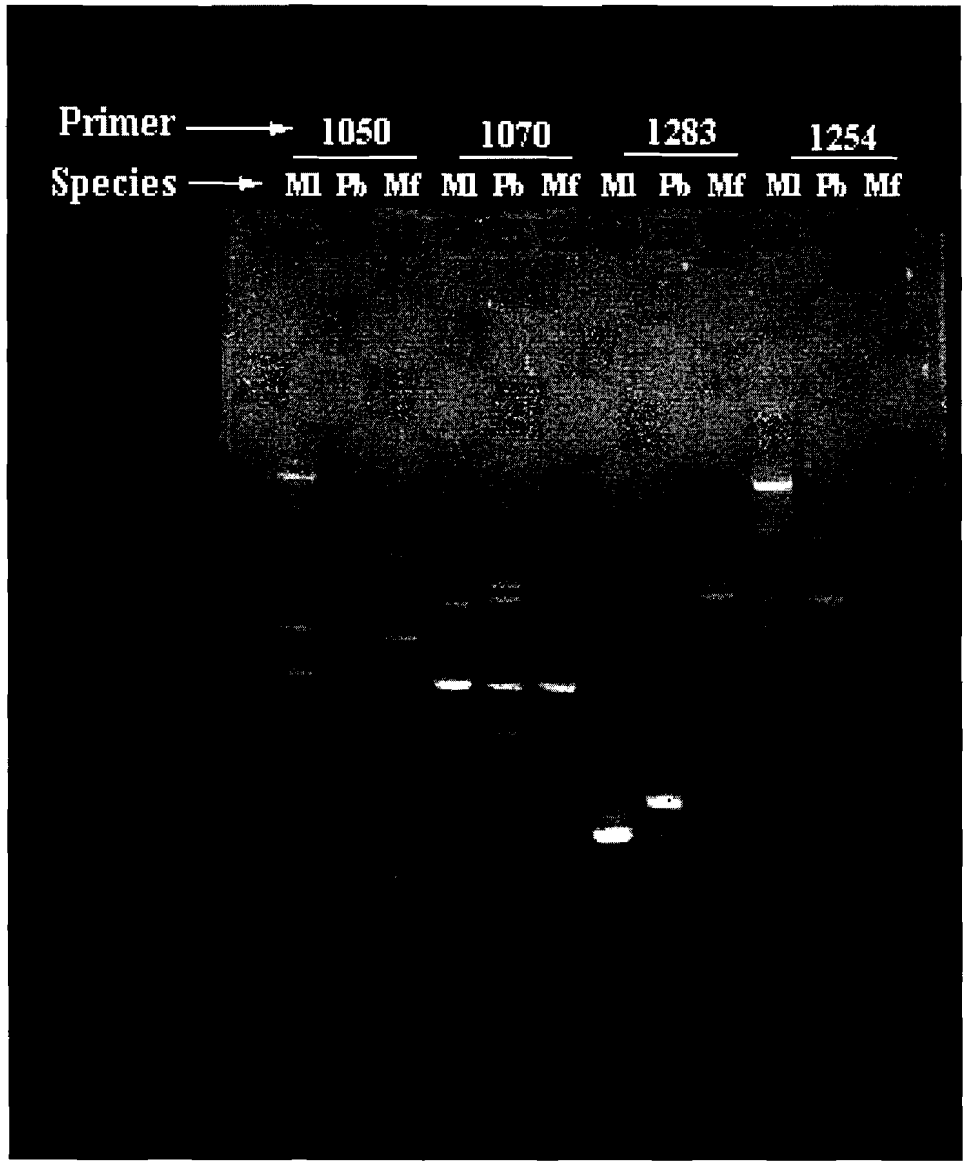

Primer
1050
1070
1283
1254
Species
Ml Pb Mf Ml Pb Mf Ml Pb Mf Ml Pb Mf

Table 1. Random primers and their reaction conditions

Primer	Sequence	Annealing temperature	Magnesium concentration	Amplification conditions [a]
1050	$^{5'}$AACAGCTCGA$^{3'}$	40° C	1.5 mM	94° C 1min 40° C 1min 72° C 1min
1070	$^{5'}$CAACCTAGCT$^{3'}$	40° C	3 mM	94° C 1 min 40° C 1min 72° C 1 min
1283	$^{5'}$GCGATCCCCA$^{3'}$	37° C	1.5 mM	94° C 1 min 37° C 1 min 72° C 1 min

[a] Amplification conditions represent 1 cycle. Each cycle was repeated a total of 35 times. An additional second was added to the annealing and extension phase with each repeat. All PCR parameters included an initial step of 94° C for 4 minutes and a final extension step at 72° C for 5 minutes.

Results

Chromosomal DNA isolation

Chromosomal DNA was isolated from the foot tissue of 82 mussels from the Neosho and Verdigris Rivers (Table 2).

Characterization using RAPD

Randomly amplified polymorphic DNA analysis was performed on 82 mussels using 3 arbitrary primers, their sequences designated 1050, 1070 and 1283 (Table 1). Primer 1050 generated 11 different sized bands as determined by agarose gel electrophoresis (Fig. 3). Primer 1070 also generated 11 different sized bands (Fig. 4), whereas primer 1283 revealed 17 different sized bands by agarose gel electrophoresis (Fig. 5). Dendrogram profiles were generated for all 3 primers testing for differences between species. The dendrograms revealed 24 groupings for primer 1050 (Fig. 6), 21 groupings for primer 1070 (Fig. 7), and 24 groupings for primer 1283 (Fig. 8). In all, these dendrograms show that RAPDs can discern differences between species and drainages. Using dendrogram analysis, Figures 9 through 17 will be used to demonstrate specific conclusions as to the applicability of RAPDs in detecting genetic diversity in these invertebrates.

Dendrogram profiles were created using all 3 primers to test for differences within species and between river drainages. Dendrogram profiles using primer 1050 revealed 12 genotypic groupings for the 45 *Q. quadrula* specimens, 7 genotypic groupings for the 19 *Q. metanevra* specimens, and 7 genotypic groupings for the 18 *Q. pustulosa* specimens

(Fig. 9 - 11). Figure 9 shows that *Q. quadrula* has biotypes with similarities between drainages and unique biotypes that appear in each drainage. For example Verdigris specimens 29 and 34 are one unique biotype, specimens 40, 41, 27 and 28 another, while 42, 30, 21 and 18 are 4 additional biotypes all unique to the Veridgris River. Whereas the Neosho River only has 2 unique biotypes, specimens 4 and 16. Both rivers have 4 common biotypes present in each drainage. Thus the diversity of *Q. quadrula* appears greater in the Verdigris River with 6 unique biotypes in all, while the Neosho only has 2. Figure 10 allows you to discern 6 biotypes and 1 minor biotype (#11) for *Q. metanvera* with primer 1050 in the Neosho River. Figure 11 shows primer 1050 discerning species diversity within *Q. pustulosa.* The Neosho drainage appears to have more diversity, with 5 unique biotypes out of the 7 total in both drainages, whereas the Verdigris does not have any unique biotypes and both rivers share 2 common biotypes.

Repeating these same questions with primer 1070 revealed 6 genotypic groupings for the 45 *Q. quadrula* specimens, 6 genotypic groupings for the 19 *Q. metanevra* specimens, and 15 genotypic groupings for the 18 *Q. pustulosa* specimens (Fig. 12 - 14). Figure 12 shows again that the Neosho River has more diversity; of the 6 total groupings, only the Neosho River biotypes show any divergence within species. While the Neosho River biotypes are present in all 6 groupings, the Verdigris specimens are only present in 3 of the groupings. Figure 13 shows that primer 1070 revealed 6 different biotypes for *Q. metanevra* in the Neosho drainage. Figure 14 reveals more diversity than seen in figures 12 and 13, revealing 15 different biotypes, 10 of which are unique to the Neosho River, 4 unique to the Verdigris River and 1 in common with both rivers. Therefore, primer 1070

seems able to best detect genetic diversity and discern unique biotypes within *Q. pustulosa* in the Neosho River, whereas primer 1050 was better able to detect diversity and discern unique biotypes within *Q. quadrula* in the Verdigris River (Figure 14 vs Figure 11).

Dendrogram profiles using primer 1283 revealed 10 genotypic groupings for the 45 *Q. quadrula* specimens, 10 genotypic groupings for the 19 *Q. metanevra* specimens, and 11 genotypic groupings for the 18 *Q. pustulosa* specimens (Fig. 15 - 17). Figure 15 shows 10 biotypes overall, with 5 unique to the Neosho River, 3 unique to the Verdigris River and 2 common in both rivers. Figure 16 shows a great deal of diversity within *Q. metanevra*, revealing 10 biotypes in the Neosho River. Figure 17 shows a total of 11 different biotypes, with 7 unique to the Neosho River, 1 unique to the Verdigris River, and 3 in common with both rivers. These data further supports the idea that the Neosho River has more genetic diversity.

Reproducibility

The reproducibility study revealed banding patterns were consistent when RAPD analysis was performed on 3 separate DNA samples isolated from the same mussel (Fig. 18).

Mark and recapture

Twenty one mussels were recaptured from the Verdigris River on April 25, 2000, 16 were *Q. quadrula* and 5 were *Q. pustulosa* (Fig. 19). Recapture efforts on the Neosho

river were not possible at that time due to high water. The second and final recapture effort took place on May 19, 2000, resulting in 4 *Q. quadrula* mussels being recaptured from the Verdigris River (recapture efforts took place approximately 4 months after the mussels were returned to the river). Twenty eight mussels were recaptured from the Neosho River during this second attempt, 12 were *Q. metanevra*, 11 *Q. quadrula* and 5 *Q. pustulosa*. Overall recapture efforts resulted in a 65% total recapture for all 82 mussels, with 56% coming from the Neosho River and a 78% from the Verdigris River. *Q. metanevra* made up the largest percentage of mussels recaptured from the Neosho River, constituting 63%, while *Q. quadrula* and *Q. pustulosa* made up 55% and 45% respectively. Recapture results from the Verdigris River revealed *Q. quadrula* had the highest recapture rate with 80%, while *Q. pustulosa* made up 71% (Table 3).

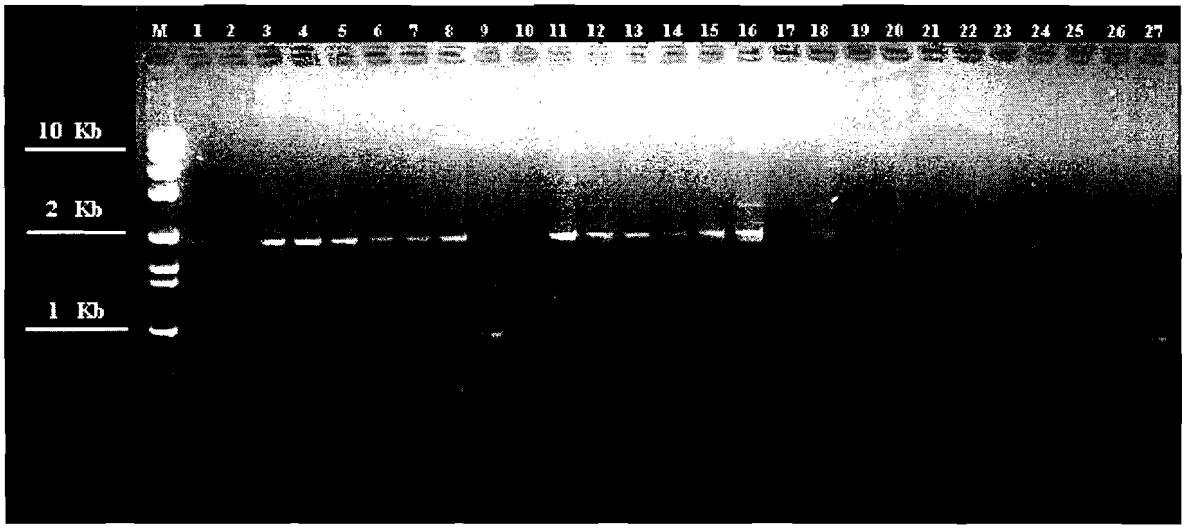
Table 2. Species and river drainage of the 82 mussels used for RAPD analysis

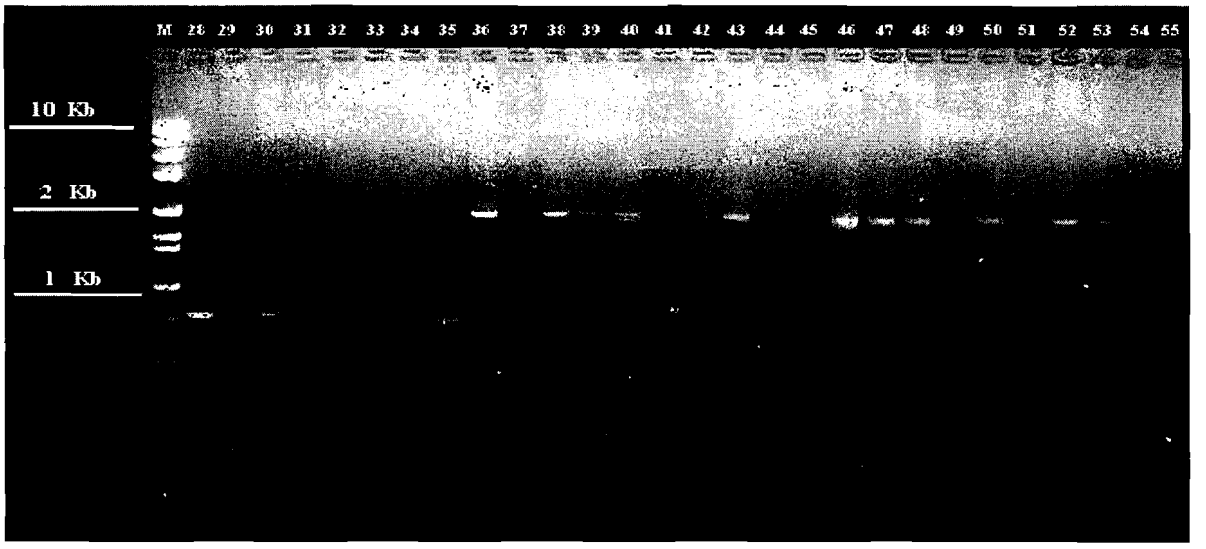
Number	Species	River Drainage
1	*Quadrula quadrula*	Neosho
2	*Quadrula pustulosa*	Neosho
3	*Quadrula quadrula*	Neosho
4	*Quadrula quadrula*	Neosho
5	*Quadrula quadrula*	Neosho
6	*Quadrula quadrula*	Neosho
7	*Quadrula quadrula*	Neosho
8	*Quadrula quadrula*	Neosho
9	*Quadrula metanevra*	Neosho
10	*Quadrula quadrula*	Neosho
11	*Quadrula quadrula*	Neosho
12	*Quadrula quadrula*	Neosho
13	*Quadrula quadrula*	Neosho
14	*Quadrula quadrula*	Neosho
15	*Quadrula quadrula*	Neosho
16	*Quadrula quadrula*	Neosho
17	*Quadrula quadrula*	Neosho
18	*Quadrula quadrula*	Neosho
19	*Quadrula quadrula*	Neosho
20	*Quadrula metanevra*	Neosho
21	*Quadrula metanevra*	Neosho
22	*Quadrula metanevra*	Neosho
23	*Quadrula metanevra*	Neosho
24	*Quadrula metanevra*	Neosho
25	*Quadrula metanevra*	Neosho
26	*Quadrula metanevra*	Neosho
27	*Quadrula metanevra*	Neosho
28	*Quadrula pustulosa*	Neosho
29	*Quadrula pustulosa*	Neosho
30	*Quadrula pustulosa*	Neosho
31	*Quadrula pustulosa*	Neosho
32	*Quadrula pustulosa*	Verdigris
33	*Quadrula pustulosa*	Verdigris
34	*Quadrula pustulosa*	Verdigris
35	*Quadrula quadrula*	Verdigris
36	*Quadrula quadrula*	Verdigris
37	*Quadrula quadrula*	Verdigris
38	*Quadrula quadrula*	Verdigris
39	*Quadrula quadrula*	Verdigris
40	*Quadrula quadrula*	Verdigris

41	*Quadrula quadrula*	Verdigris
42	*Quadrula quadrula*	Verdigris
43	*Quadrula quadrula*	Verdigris
44	*Quadrula pustulosa*	Verdigris
45	*Quadrula pustulosa*	Verdigris
46	*Quadrula quadrula*	Verdigris
47	*Quadrula quadrula*	Verdigris
48	*Quadrula quadrula*	Verdigris
49	*Quadrula quadrula*	Verdigris
50	*Quadrula quadrula*	Verdigris
51	*Quadrula quadrula*	Verdigris
52	*Quadrula quadrula*	Verdigris
53	*Quadrula quadrula*	Verdigris
54	*Quadrula quadrula*	Verdigris
55	*Quadrula quadrula*	Verdigris
56	*Quadrula metanevra*	Neosho
57	*Quadrula metanevra*	Neosho
58	*Quadrula metanevra*	Neosho
59	*Quadrula pustulosa*	Neosho
60	*Quadrula pustulosa*	Neosho
61	*Quadrula pustulosa*	Neosho
62	*Quadrula metanevra*	Neosho
63	*Quadrula metanevra*	Neosho
64	*Quadrula metanevra*	Neosho
65	*Quadrula metanevra*	Neosho
66	*Quadrula metanevra*	Neosho
67	*Quadrula metanevra*	Neosho
68	*Quadrula pustulosa*	Neosho
69	*Quadrula pustulosa*	Neosho
70	*Quadrula pustulosa*	Neosho
71	*Quadrula quadrula*	Neosho
72	*Quadrula quadrula*	Neosho
73	*Quadrula quadrula*	Neosho
74	*Quadrula metanevra*	Neosho
75	*Quadrula pustulosa*	Verdigris
76	*Quadrula pustulosa*	Verdigris
77	*Quadrula quadrula*	Verdigris
78	*Quadrula quadrula*	Verdigris
79	*Quadrula quadrula*	Verdigris
80	*Quadrula quadrula*	Verdigris
81	*Quadrula quadrula*	Verdigris
82	*Quadrula quadrula*	Verdigris

Numbers 1-82 correspond with figures 3-8.

Fig. 3. 1.5 % agarose gels of RAPD PCR products from all 82 mussels using primer 1050. DNA markers (M) are labeled and present in the first lane of all 3 gels. Lane numbers represent specimens 1-27, 28-55 and 56-82. Agarose gel electrophoresis revealed 11 different sized bands.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
10 Kb
2 Kb
1 Kb


M 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55
10 Kb
2 Kb
1 Kb

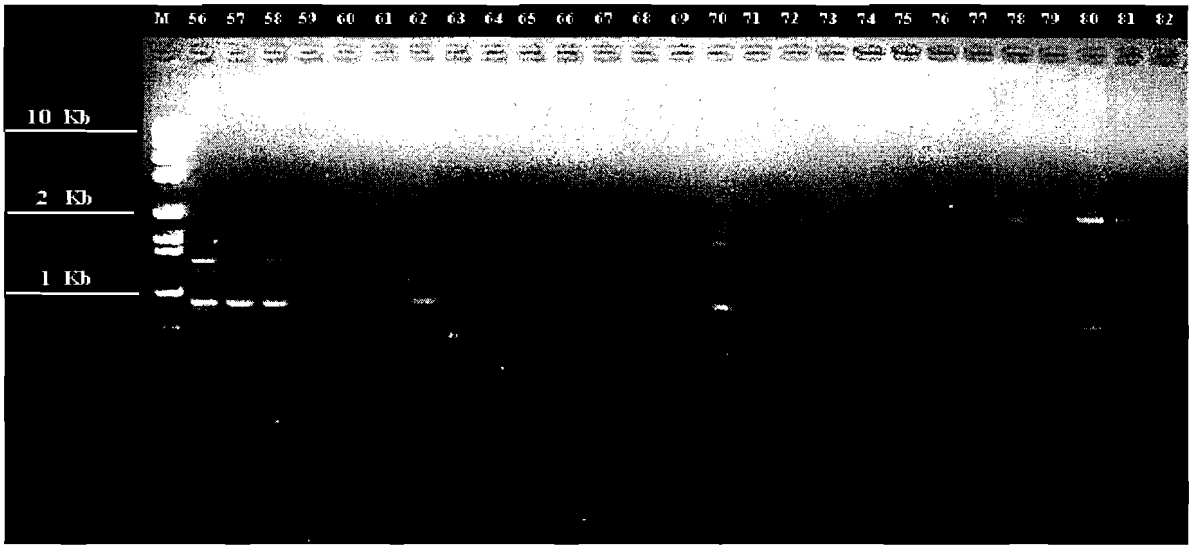
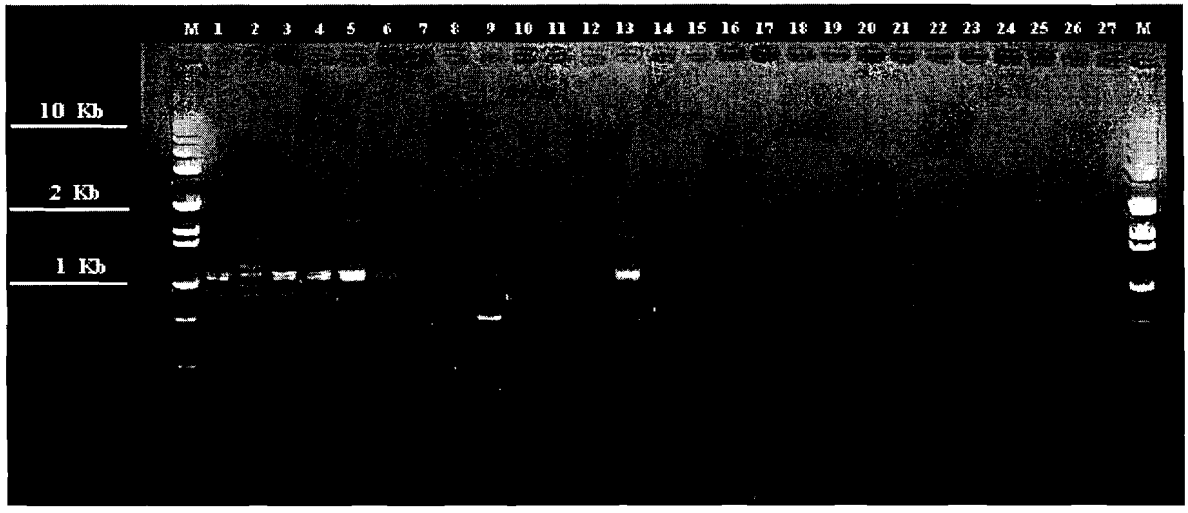
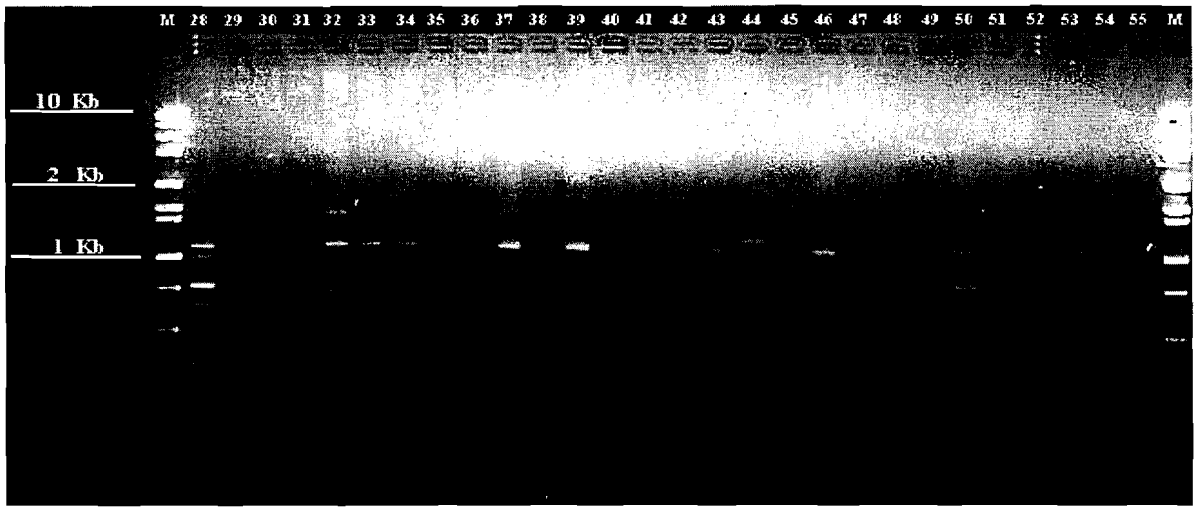

M 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82
10 Kb
2 Kb
1 Kb

Fig. 4. 1.5 % agarose gels of RAPD PCR products from all 82 mussels using primer 1070. DNA markers (M) are labeled and present in the first and last lane of all 3 gels. Lane numbers represent specimens 1-27, 28-55 and 56-82. Agarose gel electrophoresis revealed 11 different sized bands.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 M
10 Kb
2 Kb
1 Kb


M 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 M
10 Kb
2 Kb
1 Kb

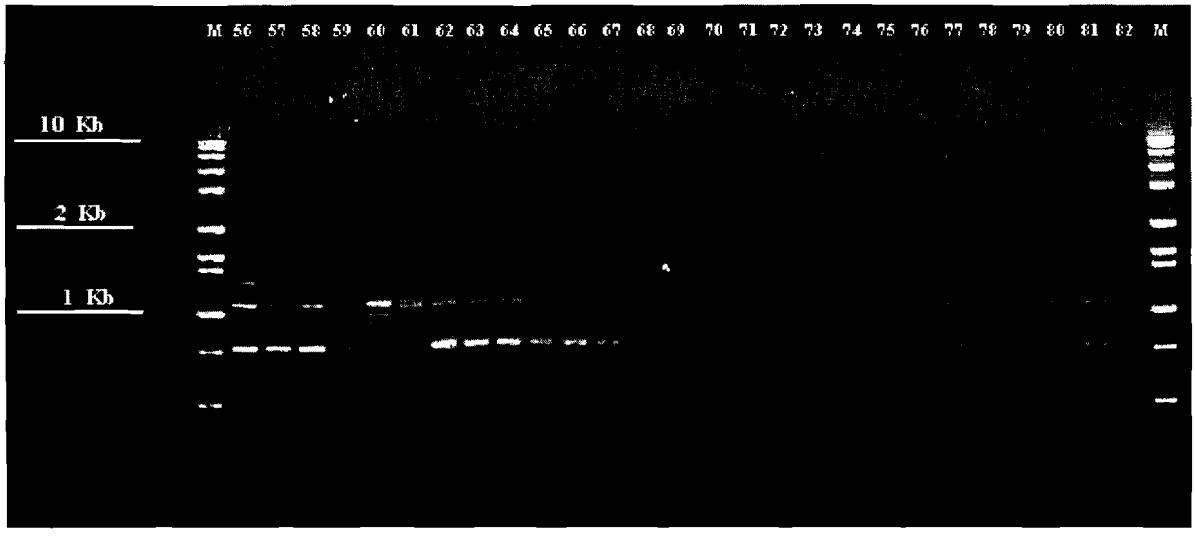
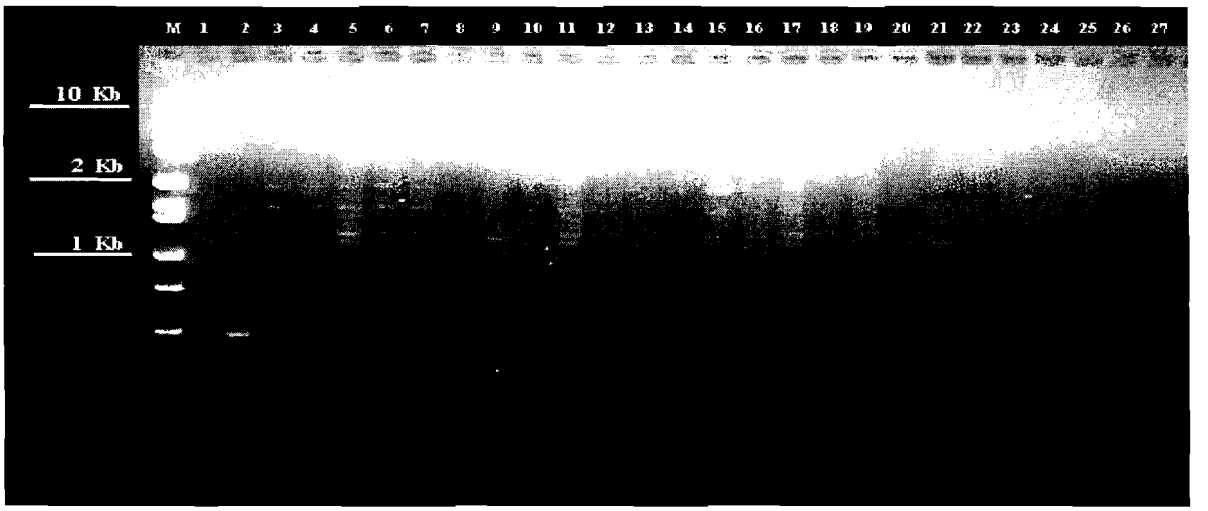
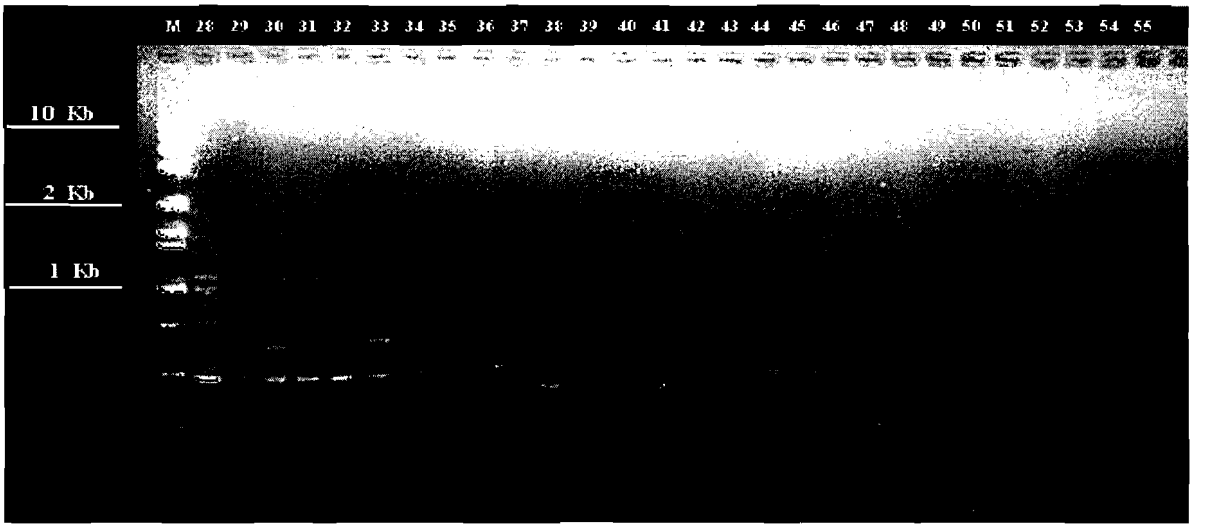

M 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 M
10 Kb
2 Kb
1 Kb

Fig. 5. 1.5 % agarose gels of RAPD PCR products from all 82 mussels using primer 1283. DNA markers (M) are labeled and present in the first lane of all 3 gels. Lane numbers represent specimens 1-27, 28-55 and 56-82. Agarose gel electrophoresis revealed 17 different sized bands.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
10 Kb
2 Kb
1 Kb


M 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55
10 Kb
2 Kb
1 Kb

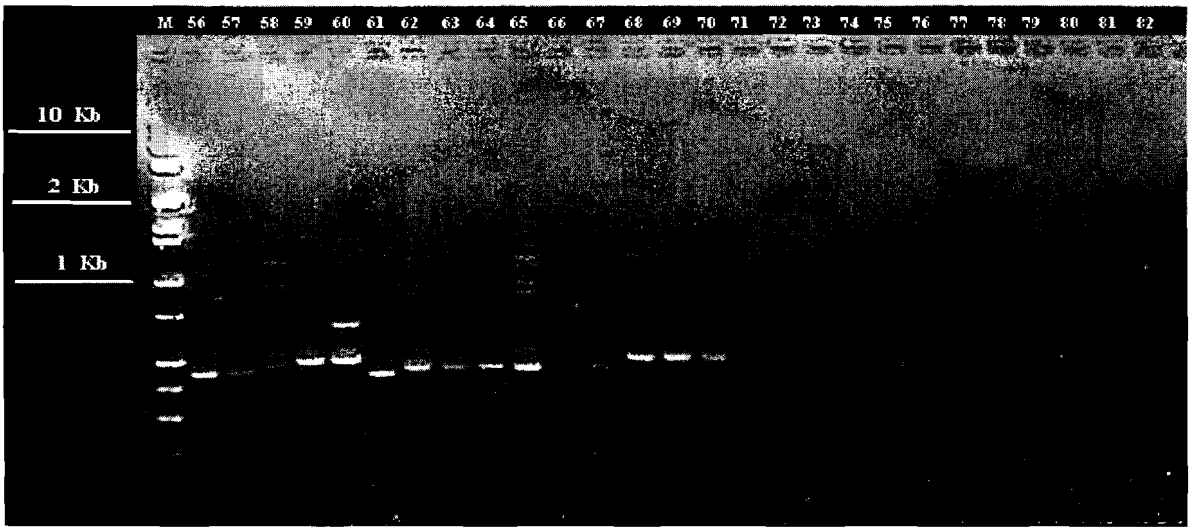

M 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82
10 Kb
2 Kb
1 Kb

Fig. 6. Dendrogram analysis of RAPD data for all 82 mussels using primer 1050. Twenty four genotypic groupings were generated. Numbers correspond to the listing in Table 2.

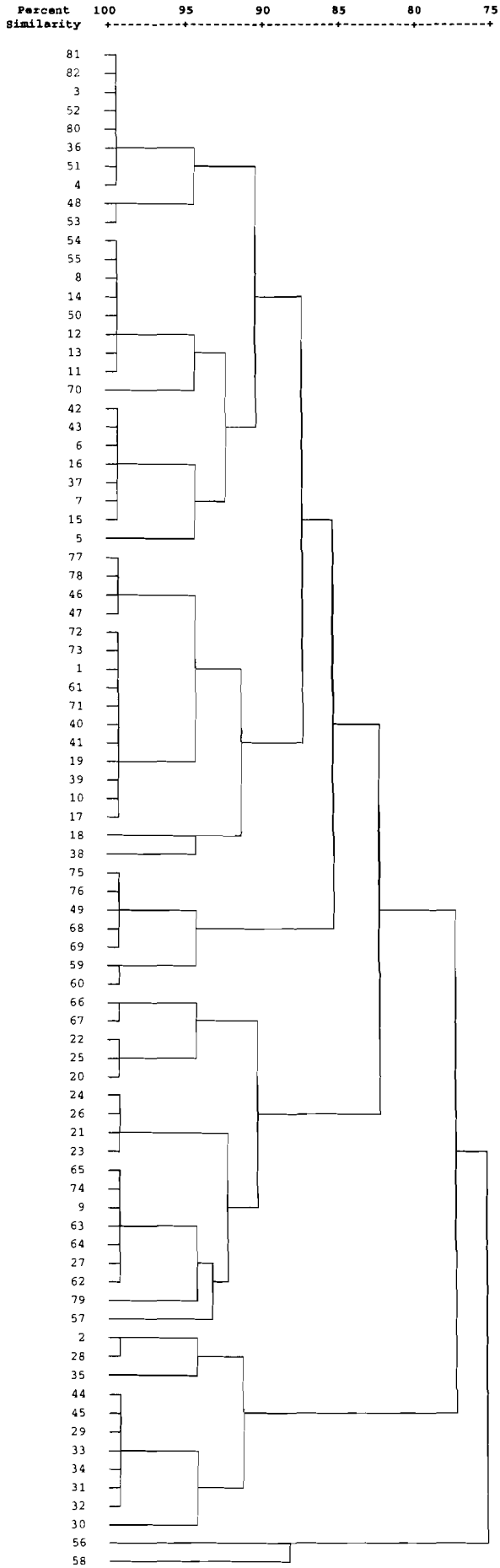

Percent Similarity
100
95
90
85
80
75
81
82
3
52
80
36
51
4
48
53
54
55
8
14
50
12
13
11
70
42
43
6
16
37
7
15
5
77
78
46
47
72
73
1
61
71
40
41
19
39
10
17
18
38
75
76
49
68
69
59
60
66
67
22
25
20
24
26
21
23
65
74
9
63
64
27
62
79
57
2
28
35
44
45
29
33
34
31
32
30
56
58

Fig. 7. Dendrogram analysis of RAPD data for all 82 mussels using primer 1070. Twenty one genotypic groupings were generated. Numbers correspond to the listing in Table 2.

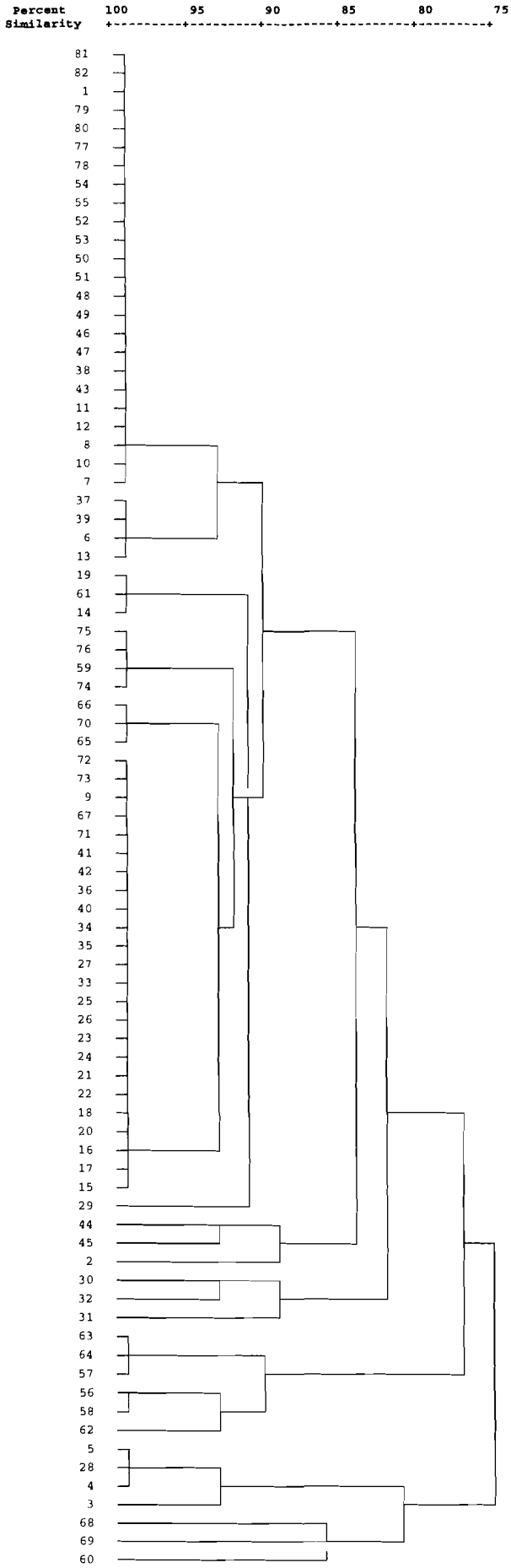

Percent Similarity
100
95
90
85
80
75
81
82
1
79
80
77
78
54
55
52
53
50
51
48
49
46
47
38
43
11
12
8
10
7
37
39
6
13
19
61
14
75
76
59
74
66
70
65
72
73
9
67
71
41
42
36
40
34
35
27
33
25
26
23
24
21
22
18
20
16
17
15
29
44
45
2
30
32
31
63
64
57
56
58
62
5
28
4
3
68
69
60

Fig. 8. Dendrogram analysis of RAPD data for all 82 mussels using primer 1283. Twenty four genotypic groupings were generated. Numbers correspond to the listing in Table 2.

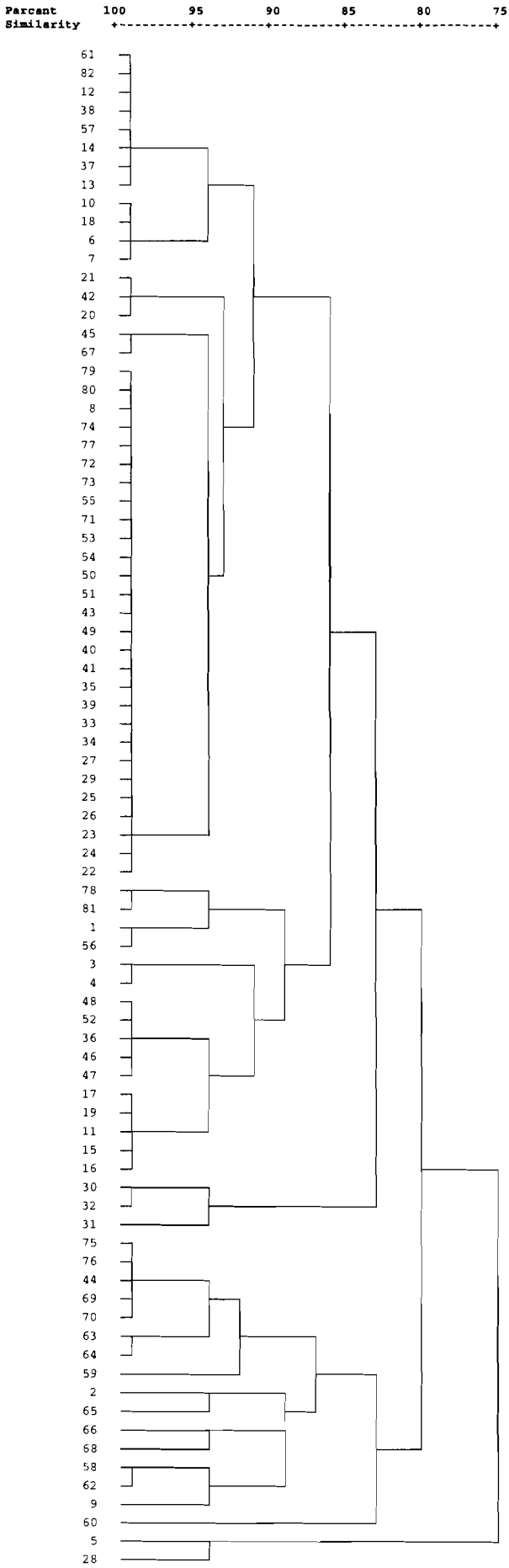

Percent Similarity
100
95
90
85
80
75
61
82
12
38
57
14
37
13
10
18
6
7
21
42
20
45
67
79
80
8
74
77
72
73
55
71
53
54
50
51
43
49
40
41
35
39
33
34
27
29
25
26
23
24
22
78
81
1
56
3
4
48
52
36
46
47
17
19
11
15
16
30
32
31
75
76
44
69
70
63
64
59
2
65
66
68
58
62
9
60
5
28

Fig. 9. Dendrogram analysis of RAPD data for all 45 *Quadrula quadrula* specimens using primer 1050. Twelve genotypic groupings were generated. Numbers refer only to the total number of *Quadrula quadrula* specimens, they do not correspond with Table 2. The N and V to the left of the numbers represent which river drainage each mussel came from, N=Neosho and V=Verdigris.

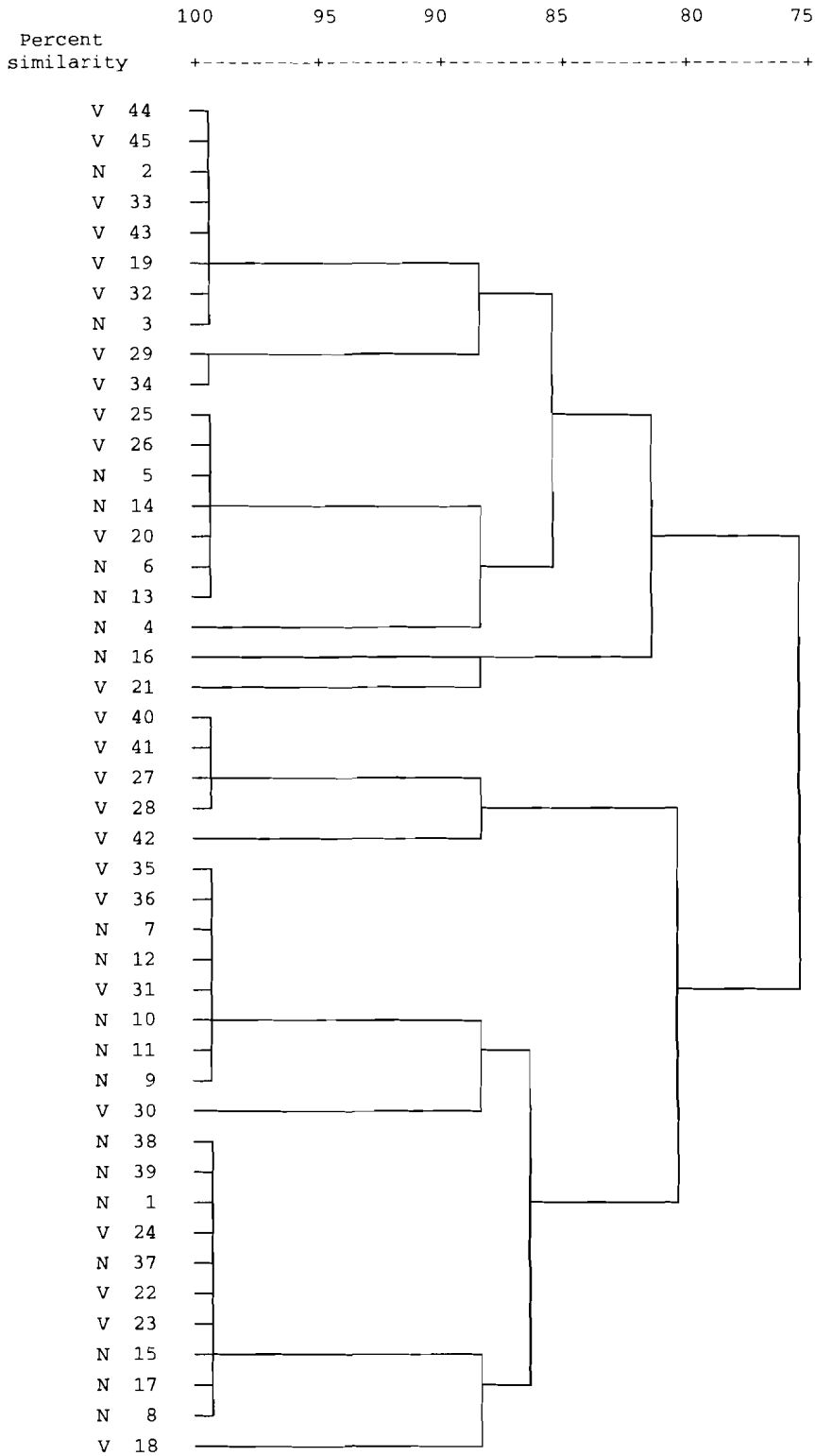

100
95
90
85
80
75
Percent
similarity
V 44
V 45
N 2
V 33
V 43
V 19
V 32
N 3
V 29
V 34
V 25
V 26
N 5
N 14
V 20
N 6
N 13
N 4
N 16
V 21
V 40
V 41
V 27
V 28
V 42
V 35
V 36
N 7
N 12
V 31
N 10
N 11
N 9
V 30
N 38
N 39
N 1
V 24
N 37
V 22
V 23
N 15
N 17
N 8
V 18

Fig. 10. Dendrogram analysis of RAPD data for all 19 *Quadrula metanevra* specimens using primer 1050. Seven genotypic groupings were generated. Numbers refer only to the total number of *Quadrula metanvera* specimens, they do not correspond with Table 2. *Quadrula metanevra* was only collected from the Neosho River.

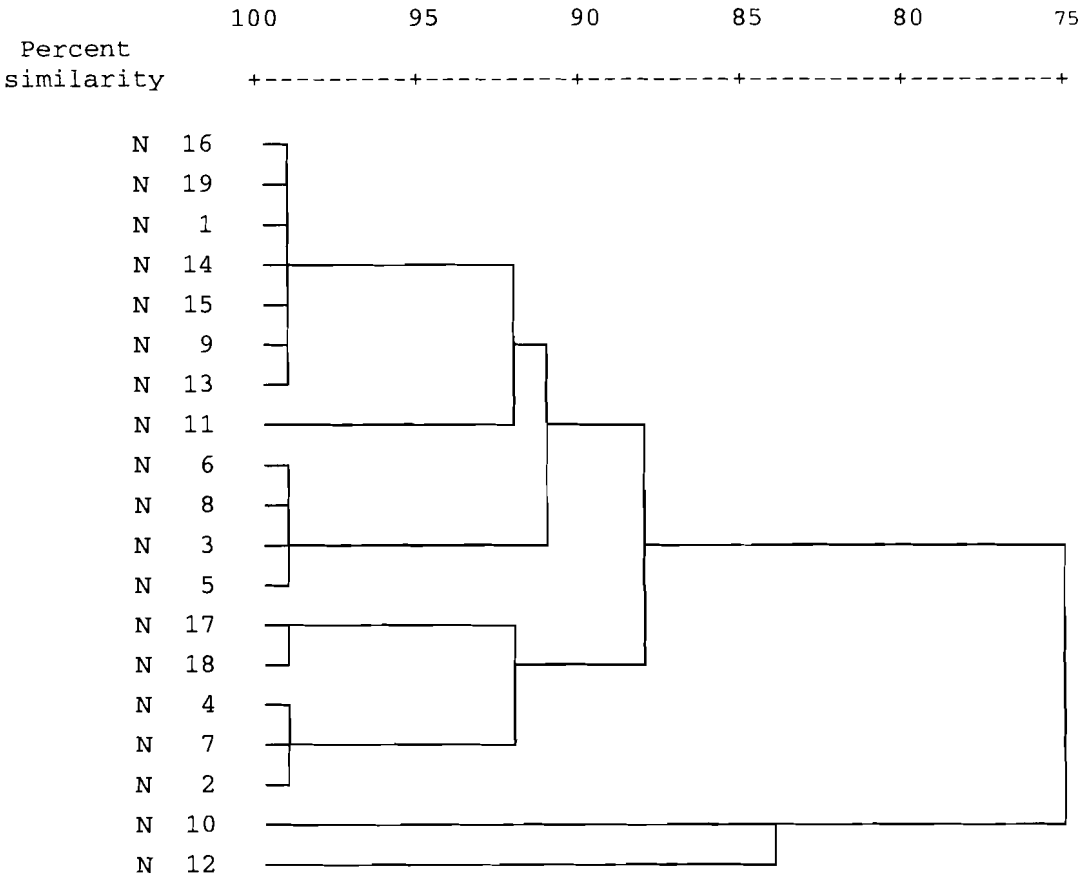

100
95
90
85
80
75
Percent
similarity
N 16
N 19
N 1
N 14
N 15
N 9
N 13
N 11
N 6
N 8
N 3
N 5
N 17
N 18
N 4
N 7
N 2
N 10
N 12

Fig. 11. Dendrogram analysis of RAPD data for all 18 *Quadrula pustulosa* specimens using primer 1050. Seven genotypic groupings were generated. Numbers refer only to the total number of *Quadrula pustulosa* specimens, they do not correspond with Table 2. The N and V to the left of the numbers represent which river drainage each mussel came from, N=Neosho and V=Verdigris.

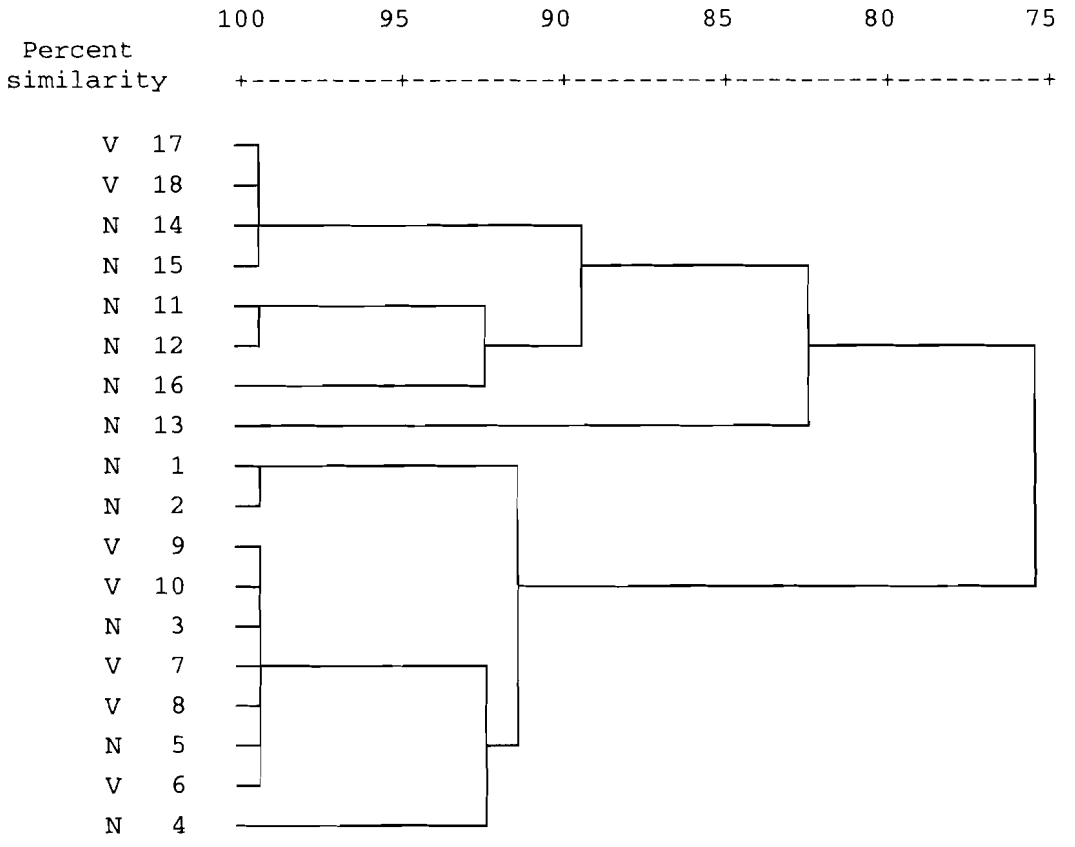

100
95
90
85
80
75
Percent
similarity
V 17
V 18
N 14
N 15
N 11
N 12
N 16
N 13
N 1
N 2
V 9
V 10
N 3
V 7
V 8
N 5
V 6
N 4

Fig. 12. Dendrogram analysis of RAPD data for all 45 *Quadrula quadrula* specimens using primer 1070. Six genotypic groupings were generated. Numbers refer only to the total number of *Quadrula quadrula* specimens, they do not correspond with Table 2. The N and V to the left of the numbers represent which river drainage each mussel came from, N=Neosho and V=Verdigris.

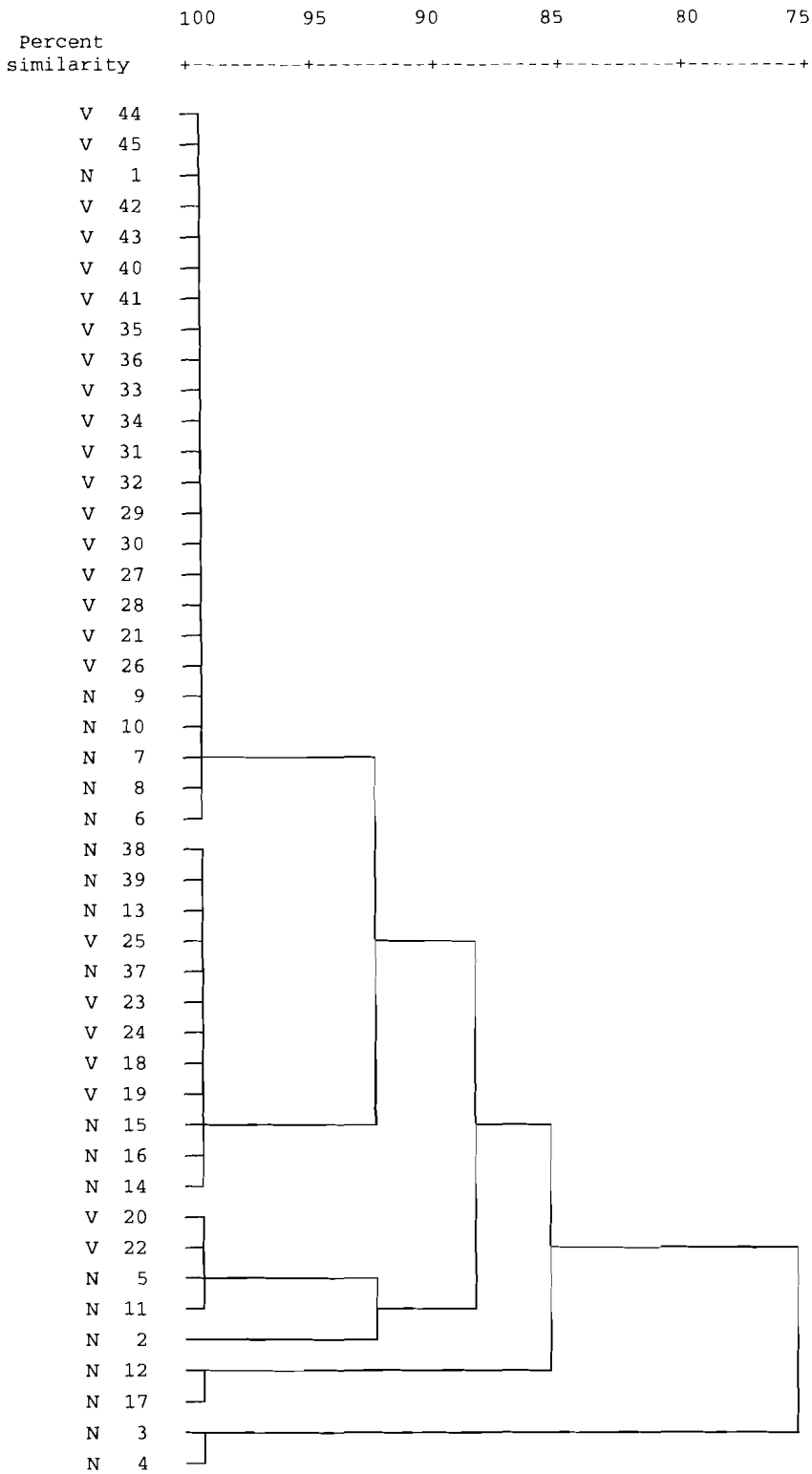

100
95
90
85
80
75
Percent similarity
V 44
V 45
N 1
V 42
V 43
V 40
V 41
V 35
V 36
V 33
V 34
V 31
V 32
V 29
V 30
V 27
V 28
V 21
V 26
N 9
N 10
N 7
N 8
N 6
N 38
N 39
N 13
V 25
N 37
V 23
V 24
V 18
V 19
N 15
N 16
N 14
V 20
V 22
N 5
N 11
N 2
N 12
N 17
N 3
N 4

Fig. 13. Dendrogram analysis of RAPD data for all 19 *Quadrula metanevra* specimens using primer 1070. Six genotypic groupings were generated. Numbers refer only to the total number of *Quadrula metanvera* specimens, they do not correspond with Table 2. *Quadrula metanvera* was only collected from the Neosho River.

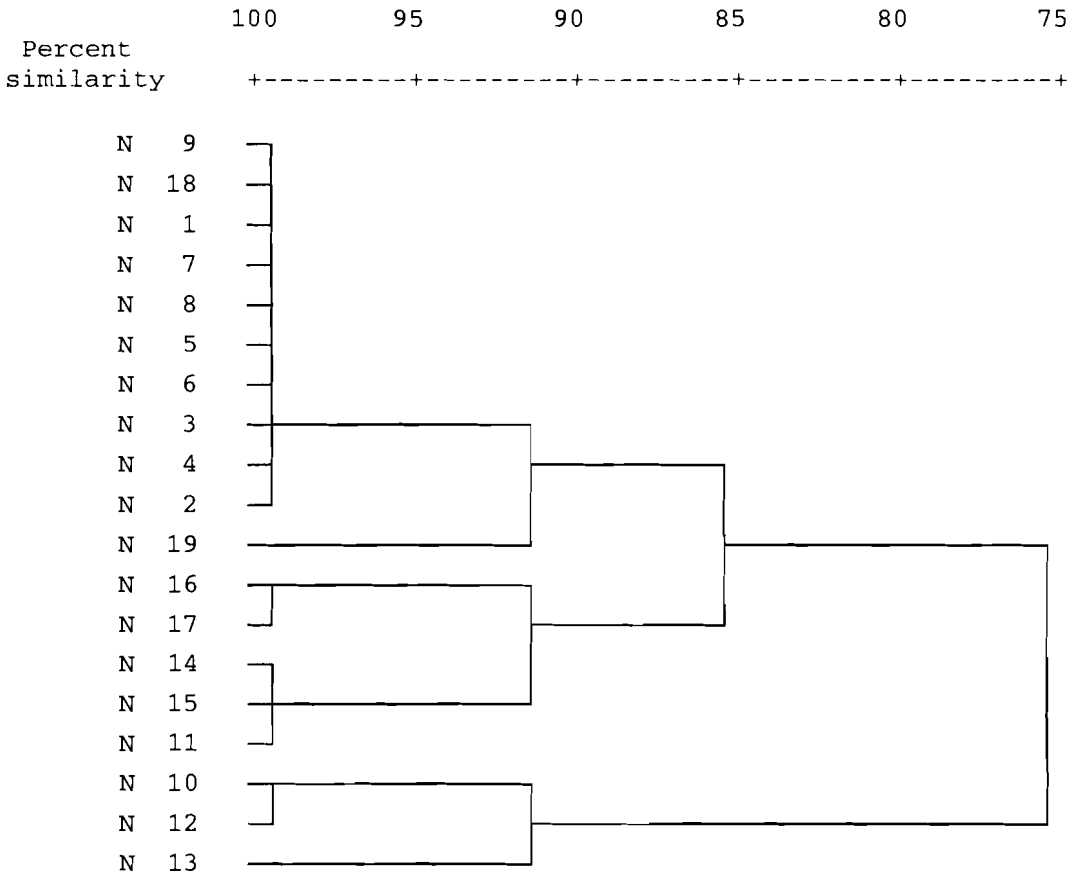

100
95
90
85
80
75
Percent
similarity
N 9
N 18
N 1
N 7
N 8
N 5
N 6
N 3
N 4
N 2
N 19
N 16
N 17
N 14
N 15
N 11
N 10
N 12
N 13

Fig. 14. Dendrogram analysis of RAPD data for all 18 *Quadrula pustulosa* specimens using primer 1070. Fifteen genotypic groupings were generated. Numbers refer only to the total number of *Quadrula pustulosa* specimens, they do not correspond with Table 2. The N and V to the left of the numbers represent which river drainage each mussel came from, N=Neosho and V=Verdigris.

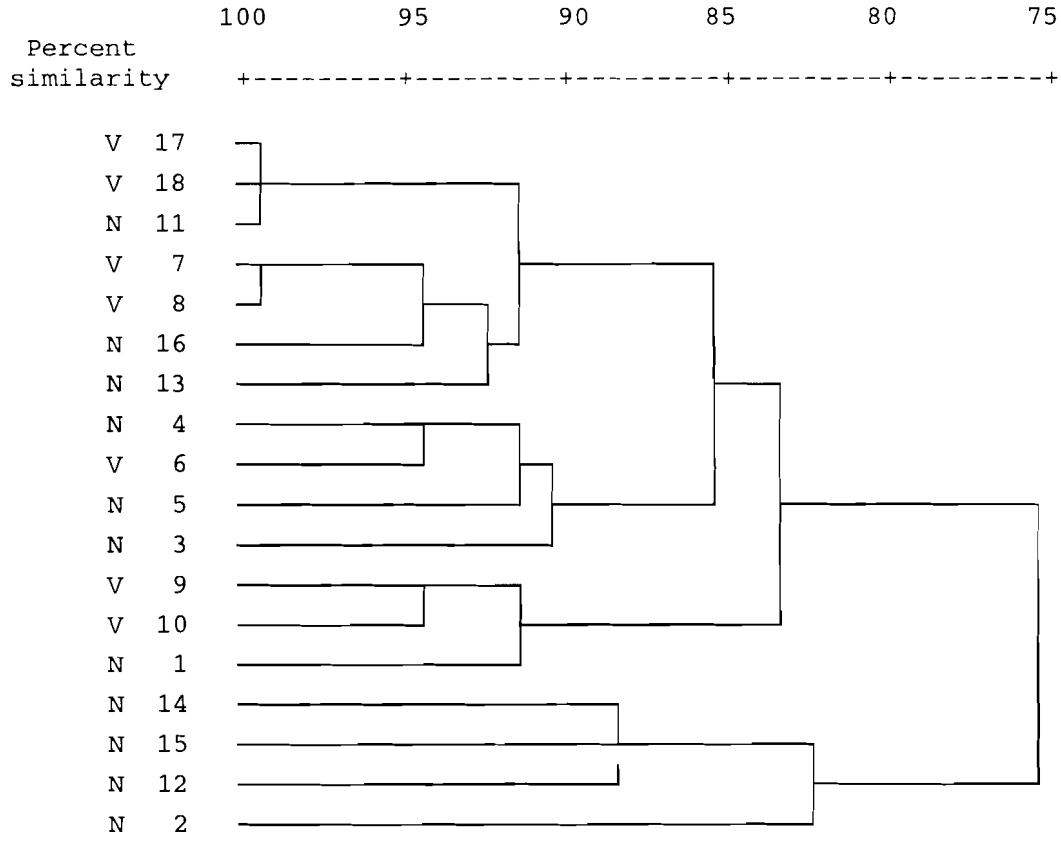

100
95
90
85
80
75
Percent
similarity
V 17
V 18
N 11
V 7
V 8
N 16
N 13
N 4
V 6
N 5
N 3
V 9
V 10
N 1
N 14
N 15
N 12
N 2

Fig. 15. Dendrogram analysis of RAPD data for all 45 *Quadrula quadrula* specimens using primer 1283. Ten genotypic groupings were generated. Numbers refer only to the total number of *Quadrula quadrula* specimens, they do not correspond with Table 2. The N and V to the left of the numbers represent which river drainage each mussel came from, N=Neosho and V=Verdigris.

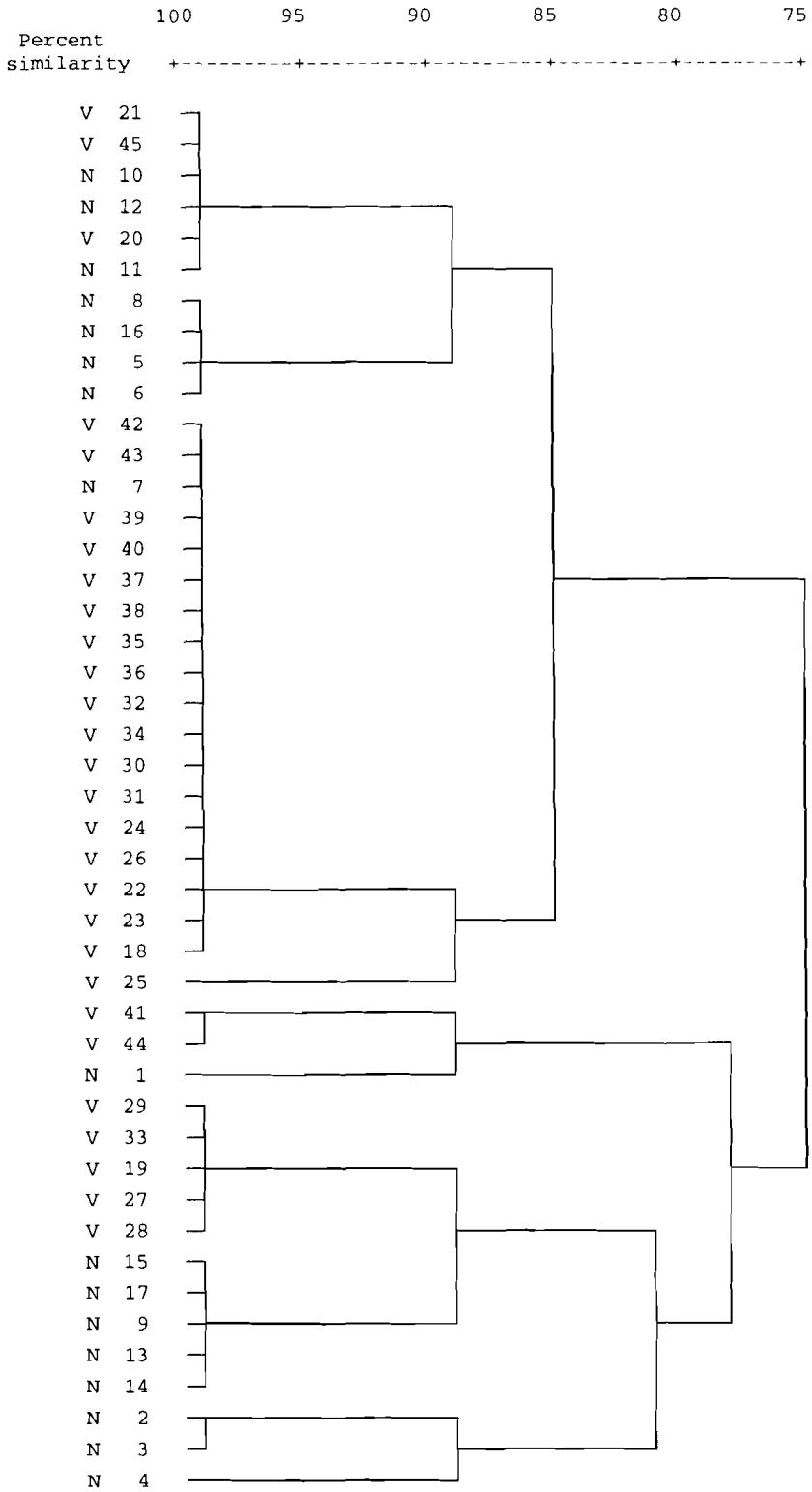

100
95
90
85
80
75
Percent
similarity
V 21
V 45
N 10
N 12
V 20
N 11
N 8
N 16
N 5
N 6
V 42
V 43
N 7
V 39
V 40
V 37
V 38
V 35
V 36
V 32
V 34
V 30
V 31
V 24
V 26
V 22
V 23
V 18
V 25
V 41
V 44
N 1
V 29
V 33
V 19
V 27
V 28
N 15
N 17
N 9
N 13
N 14
N 2
N 3
N 4

Fig. 16. Dendrogram analysis of RAPD data for all 19 *Quadrula metanevra* specimens using primer 1283. Ten genotypic groupings were generated. Numbers refer only to the total number of *Quadrula metanvera* specimens, they do not correspond with Table 2. *Quadrula metanvera* was only collected from the Neosho River.

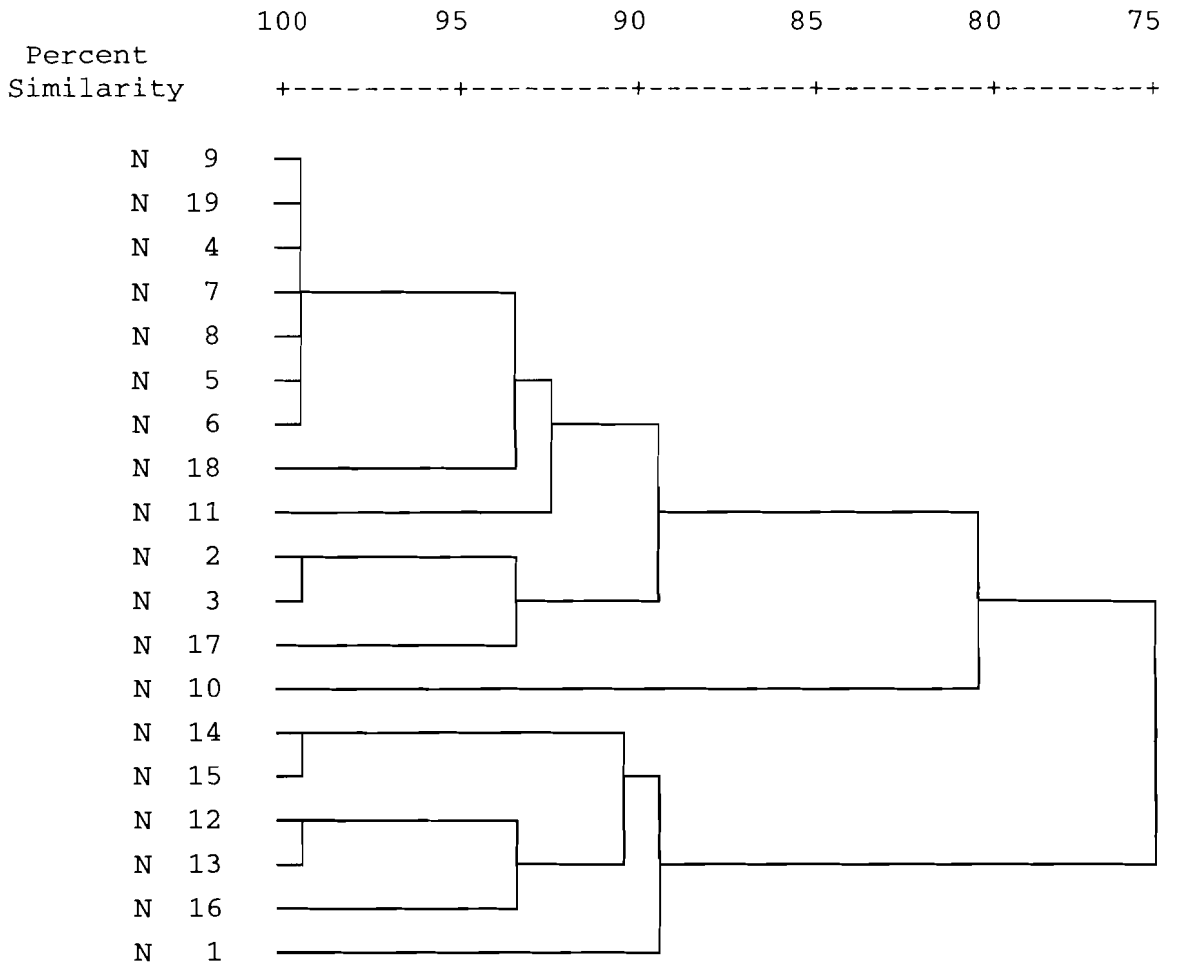

100
95
90
85
80
75
Percent
Similarity
N 9
N 19
N 4
N 7
N 8
N 5
N 6
N 18
N 11
N 2
N 3
N 17
N 10
N 14
N 15
N 12
N 13
N 16
N 1

Fig. 17. Dendrogram analysis of RAPD data for all 18 *Quadrula pustulosa* specimens using primer 1283. Eleven genotypic groupings were generated. Numbers refer only to the total number of *Quadrula pustulosa* specimens, they do not correspond with Table 2. The N and V to the left of the numbers represent which river drainage each mussel came from, N=Neosho and V=Verdigris.

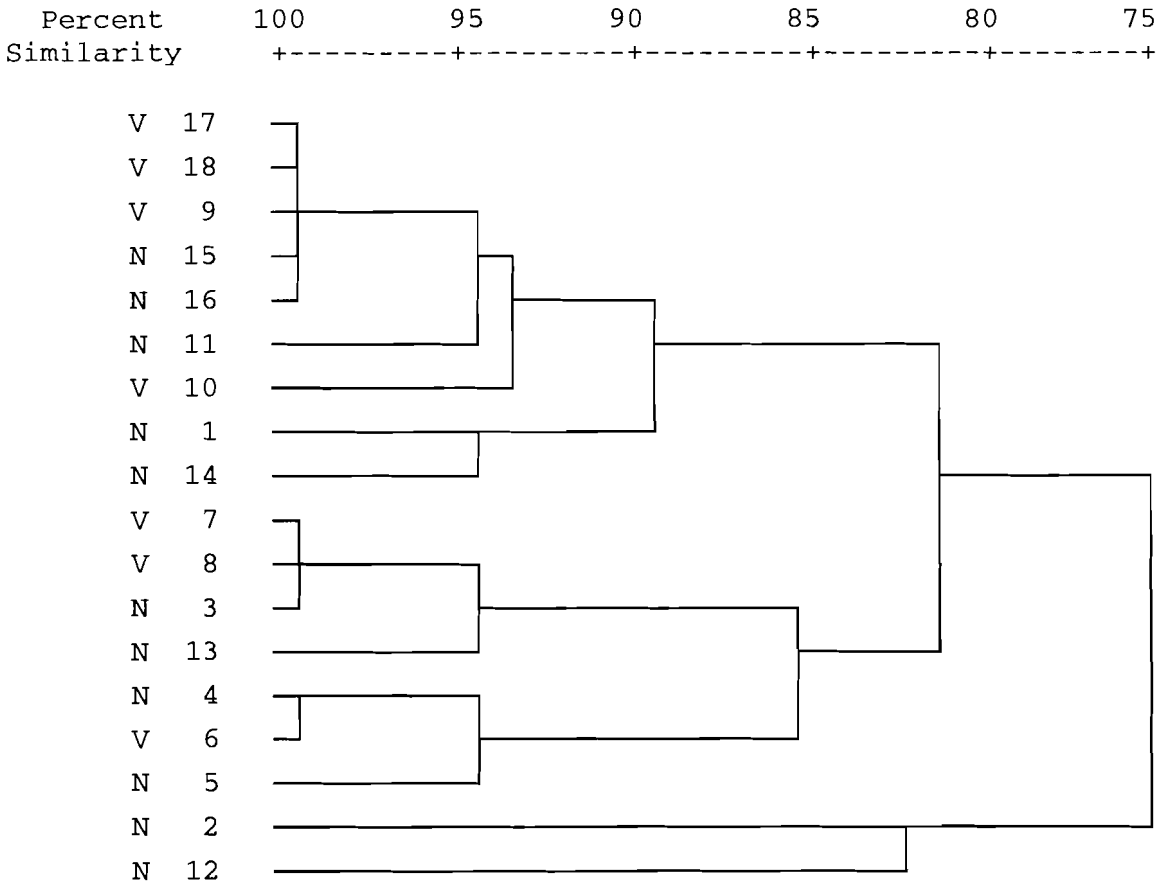

Percent Similarity
100
95
90
85
80
75
V 17
V 18
V 9
N 15
N 16
N 11
V 10
N 1
N 14
V 7
V 8
N 3
N 13
N 4
V 6
N 5
N 2
N 12

Fig. 18. Reproducibility study to validate band pattern analysis. 1.5 % agarose gel of amplified DNA products from 2 different species of mussels following repeated DNA isolations. Lanes A, B and C represent 3 separate DNA isolations for each species. Two different species of mussels were used totaling 6 DNA isolations. All 6 amplified DNA products came from the same PCR reaction using primers 1050 and 1070. Consistent banding patterns for each species confirm reproducible results.

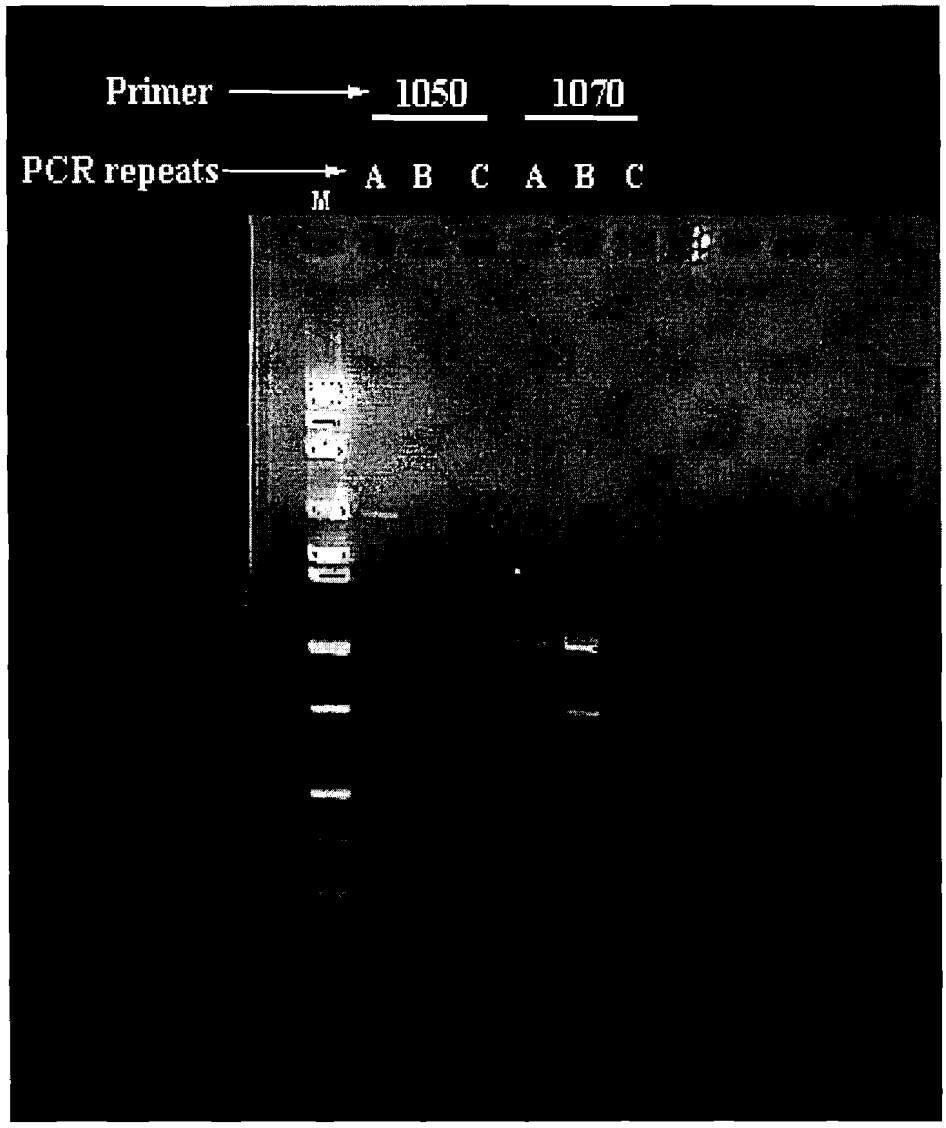

Primer
1050
1070
PCR repeats
A B C A B C
M

Fig. 19. Picture of mark and recapture efforts from the Verdigris River. The 21 mussels pictured here were recaptured approximately 4 months after being returned to the Verdigris River. All 21 were buried deep in the substrate, a testimony to their recovery. This was the first of 2 recapture efforts to take place in the Verdigris and Neosho Rivers.

Table 3. Recapture results for individual species [a]

River drainage	Species	Initial capture totals	Recapture totals	Recapture percentage
Neosho	*Q. quadrula*	20	11	55%
	Q. metanevra	19	12	63%
	Q. pustulosa	11	5	45%
Verdigris	*Q. quadrula*	25	20	80%
	Q. pustulosa	7	5	71%

[a] *Q. Quadrula* had the highest recapture percentage at the Verdigris site, *Q. metanevra* the highest at the Neosho site, while *Q. pustulosa* had the lowest recapture percentage at both sites. Interestingly, *Q. pustulosa* was the smallest species on average at both sites perhaps making it more difficult to locate during recapture efforts. Whereas *Q. quadrula* and *Q. metanvera* were the largest species on average at each of their respective sites.

Discussion

Despite a growing awareness for mussel conservation, efforts to preserve their natural habitat and understand genetic diversity continue to receive little attention. Plagued by years of pollution and habitat degradation, many species face population reductions and potential extirpation from the region and state.

Traditionally, genetic research required killing study organisms to acquire enough tissue for analysis. Now, however, with advancements in science, PCR based techniques offer researchers a nonlethal approach to understanding genetic diversity when conservation is as important as genetic analysis.

Three random primers designated 1050, 1070 and 1283 were used to determine genetic variation between mussels in the Neosho and Verdigris Rivers. No primer could absolutely discern between drainages or species although some groupings turned out to be unique. Dendrogram profiles using all 82 specimens revealed similar results in terms of numbers of groupings with primers 1050 and 1283, both having 24 groupings, while primer 1070 revealed 21 groupings. Primer 1050 detected the most diversity within the *Q. quadrula* specimens, revealing 12 genotypic groupings. Primer 1283 detected 10 genotypic groupings within *Q. metanvera*, while primer 1070 revealed the most variation within *Q. pustulosa* with 15 genotypic groupings. Primers 1070 and 1283 best distinguished Neosho River biotypes of *Q. pustulosa*. Primer 1070 was able to distinguish 10 unique Neosho River biotypes out of the 15 total groupings. Whereas primer 1283 was able to discern 7 distinct biotypes unique to the Neosho River out of the 11 groupings. Primer 1050 was able to best discern unique Verdigris River biotypes of

Q. quadrula, distinguishing 6 biotypes in the Verdigris River and only 2 unique Neosho biotypes. Primer 1050 was also able to distinguish 5 unique biotypes of *Q. pustulosa* in the Neosho River, but was unable to distinguish any unique Verdigris River biotypes. All primers were not able to discern between drainages and species to an equal degree. Distinction appeared to be based on each primer's ability to detect the genetic differences associated with separate drainages and individual diversity. The absence of *Q. metanevra* from the Verdigris River prevented their use in this study. Although once prevelant in this stretch of the Verdigris River, *Q. metanevera* has experienced a reduction in range. Range reductions reduce population numbers and therefore effect gene flow and genetic diversity. Supporting the findings of this study that there may be decreasing genetic diversity within the Verdigris River. Table 4 summarizes these findings.

Reproducibility

Reproducibility and inconsistency have long been a criticism of RAPD analysis. However, in this study and many others, standardizing conditions all but eliminated problems with repeatability (14). Reproducibility was confirmed in this study after 3 separate DNA isolations from 2 different mussels yielded consistent banding patterns using primers 1050 and 1070. This confirmed not only the repeatability of RAPD analysis, but the overall validity of the nonlethal DNA isolation technique developed in this study.

Neosho and Verdigris basins

Located in the tall grass prairie, the Neosho and Verdigris basins contain the highest diversity of freshwater mussels in Kansas and the best habitat for host fishes (10). Both rivers had several top 20 rankings in the KDHE most diverse Unionid database. Rankings were based on conservation targeting score, total taxa, and rare taxa (Score = historical/28 + current/historical + endangered x 0.3 + threatened x 0.2 + species in need of conservation x 0.1). My sampling site at Leroy, KS in the Neosho River ranked 4th overall with a score of 3.59. While there was not a score and rank for my sampling site at Madison, a rank of 11th and a score of 2.37 was given for the Verdigris River at Virgil, 20 km downstream from Madison (6).

The Neosho and Verdigris Rivers both show variation, however, comparing both drainages with all 3 primers suggests the Neosho River has more diversity. Only on one occasion when using primer 1050 with *Q. quadrula* did the Verdigris River have more unique biotypes than the Neosho. The other 5 comparisons made with the remaining primers and species revealed the Neosho River has more unique biotypes. However, even more notable was the fact that primer 1070 revealed 15 groupings for *Q. pustulosa*, the most groupings by any primer. At the same time only 1 common biotype was revealed between both drainages, whereas the Neosho River alone had 10 unique biotypes (Table 4). Does this suggest a potential bottlenecking effect or simply genetic differences associated with each drainage? Perhaps it is simply a manifestation of the decline in diversity as you move upstream, especially above impoundments. This significance may never be completely understood without larger databases analyzing genetic diversity.

Complete sampling of all drainages in the state, as well as complete drainage surveys would add to a database of mussel variability that would be a valuable future resource. This would allow complete analysis which not only provides a starting database of relationships, but also can be used to make determinations at subsequent intervals of time, for example, every 5 years. This approach would also be an excellent way to determine the environmental impact of an "event" should a die-off occur. One could sample the remaining populations to determine to what extent it has been bottlenecked by such an event.

Although both basins seem to support diverse populations of mussels, overgrazing, feedlot runoff, and pollution have changed these rivers once described as a "splendid clear water stream" in 1912, to waterways brimming with fecal coliform, pesticides and heavy metals (10). With cattle pens on one side and cropland on the other, my site on the Verdigris River represented a catch basin for pesticides and feedlot runoff. At Leroy, the river banks were cluttered with garbage and debris, while the river itself appeared to be a dumping site for carpet and trash. The damage from pollution is so great in certain areas of both rivers that Kansas Department of Wildlife and Parks (KDWP) warns anglers against eating the fish they catch. The prairie that once served as a buffer zone between diversified habitats is now trampled down and overgrazed all the way up to the river banks. City dams and federal reservoirs now block the natural migration of host fish necessary for mussel reproduction (10).

Gene flow and fish mediated dispersal

Characterizing Unionidae genetic diversity is complicated by a life cycle that requires an intermediate fish host. Glochidia released from the excurrent siphons of fertilized females attach to the fins and gills of unsuspecting fish. There they develop into juvenile mussels before dropping off into the substrate where they mature into adults. However, because each species may require a different host fish, gene dispersal can be influenced by the availability and density of fish populations as well as mobility of the host species (4). Therefore, gene flow and diversity can depend on healthy populations of native fishes. For instance, it has been shown that freshwater drum and channel catfish, both prevalent in Kansas streams, are much more mobile than longear sunfish and yellow bullheads that tend to be more prominent in ponds and lakes (12). In addition gene flow can be interrupted by dams and reservoirs which block the natural migration of host species. These factors combined with potential inbreeding, bottlenecking, selection and habitat degradation make understanding genetic diversity an issue for conservationists, population geneticists and fisheries biologists alike (4).

Recapture efforts

Recapture results for the Verdigris River were higher overall 78%, compared with the Neosho River, 56%. However, only 1 recapture effort was possible in the Neosho River due to high water, whereas 2 recapture efforts took place in the Verdigris River. Another contributing factor to differences in recapture percentages may be attributed to size differences between both rivers. The Neosho River at Leroy is considerably larger

than the Verdigris River at Madison, therefore complicating recapture efforts. Recapture efforts for individual species revealed *Q. Quadrula* had the highest recapture percentage at the Verdigris site 80%, *Q. metanevra* the highest at the Neosho site 63%, while *Q. pustulosa* had the lowest recapture percentage at both sites, 45% at the Neosho and 71% at the Verdigris. Interestingly, *Q. pustulosa* was the smallest species on average at both sites making it more difficult to locate during recapture efforts. Whereas the larger *Q. quadrula* and *Q. metanvera* were easier to locate at each of their respective sites. Eighty-two of the 85 mussels captured for this study were returned alive to each respective drainage. Recapture percentages represent the number of mussels that were located after several months in the river. Not necessarily a survival rate, but proof that the developed technique did not prevent survival once returned to their natural habitat. Considering the amount of time in captivity, tissue biopsy, flooding and possible predation after being returned to the river, total recapture percentages of 78% and 56% were remarkable.

Overview

RAPD analysis combined with a nonlethal tissue biopsy proved to be a dependable and powerful tool for detecting genetic variation in freshwater mussels. Easy to use and relatively inexpensive this procedure is a great asset to researchers practicing conservation while studying population genetics. This technique is also recommended for other species on the brink of concern. Reproducible and highly delineated this approach provides important genetic information while practicing conservation.

Caution is needed in choosing the best suited primer for detecting variability with PCR RAPDs. If this strategy was applied to fish, birds or reptiles, adequate laboratory development of ideal PCR conditions would be necessary. To this end, specific technology helps accomplish the evaluation. The Jones Biotechnology Laboratories where this work was performed had an Eppendorf Gradient Mastercycler capable of evaluating annealing temperatures of primers through a continuous temperature gradient. This saves much time and effort by answering the question of optimal amplification conditions in a single day rather than 2-3 weeks of daily, multiple experiments.

The value of this approach is also shown when compared with past procedures used for mussel assessments. Seitman's work using allozyme analysis found little genetic variation between 2 populations of mussels in the Neosho and Cottonwood Rivers, whereas RAPD analysis revealed distinct biotypes within the Neosho and Verdigris Rivers, and a large degree of diversity within each species (13). This supports the idea that RAPD analysis is indeed more sensitive than allozyme analysis in delineating genetic differences between populations. Differences that may help determine the stability of future populations. However, because of the randomness of these primers it is possible that in this or any other RAPD study introns or other biologically insignificant sites are being primed. Therefore, it is important to use multiple primers, as was the case in this study to increase the odds of accurate genetic characterization.

Although this nonlethal technique was developed for use with mussels, surveys of birds could be done the same way by exploring DNA extractions from feather follicles. While RAPD analysis was the chosen method in this study, any PCR based study

focusing on conservation or requiring small amounts of tissue would be suitable with this technique.

Table 4. Primers discerning unique biotypes and groupings with each species

Species	primer	unique NR biotypes	unique VR biotypes	common biotypes	total groupings
Q. quadrula	1050	2	6	4	12
	1070	3	0	3	6
	1283	5	3	2	10
Q. metanvera	1050	7	NA	NA	7
	1070	6	NA	NA	6
	1283	10	NA	NA	10
Q. pustulosa	1050	5	0	2	7
	1070	10	4	1	15
	1283	7	1	3	11

NA = Not applicable because *Q. metanvera* was only collected from the Neosho River.
NR biotypes=Neosho River biotypes and VR biotypes=Verdigris River biotypes

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