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An Evaluation of Hemolymph Extraction as a Non-Lethal Sampling Method for Genetic Identification of Freshwater Mussel Species in Southeastern North Carolina

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16. Abstract

Identification of freshwater mussel species and subpopulations is challenging due to the plasticity of morphological characters. Molecular analyses are a powerful alternative for systematics and for biological surveys and restoration efforts. Yet most molecular methods require sacrificing animals for tissue sampling, an approach that cannot be applied to threatened and endangered species. This study demonstrated, through field trials with 3 endemic yet abundant Lake Waccamaw mussel species, that hemolymph extraction produced no mortality in animals in their native environment. We developed extraction methods that make hemolymph a reliable source for DNA analysis that, despite lower DNA yield, shows percent success rate in polymerase chain reaction (PCR) assays that rivals that of body tissues from sacrificed animals. We followed sampled and control mussels in field enclosures in Lake Waccamaw for 8 weeks and observed no mortality of either treatment group. A small, statistically not significant reduction in growth (5 - 8%) was observed in the hemolymph-removal group. We report the development and application of PCR assays from two mitochondrial DNA (mtDNA) regions, followed with digestion by restriction enzymes that reveal species-diagnostic DNA restriction fragment length polymorphisms (RFLP). The results are rapid and inexpensive PCR-RFLP assays to discriminate among sibling species in North Carolina that are difficult and sometimes misidentified in the field, even by experienced biologists. Our regional sampling and phylogenetic analysis of mtDNAs indicates that the major taxa in Lake Waccamaw are not strictly endemic to the Lake. Samples collected in the Lake showed mtDNA sequences that were identical or very similar to sequences obtained from other rivers within the Yadkin-PeeDee and Lumber drainages. Lake Waccamaw *Elliptio wacamawensis* shared haplotypes with, and/or fell within a clade of, mussels identified as *E. congaraea* and *E. wacamawensis* from the Waccamaw River. The same was true of *Lampsilis fullerkati* specimens, which were in a clade intermingled with *L. radiata* and *L. radiata radiata* specimens from the Waccamaw and Yadkin-PeeDee rivers. Additional sampling, morphological and molecular analysis, particularly of type specimens, is needed to further resolve taxonomic relationships and biogeographic ranges. A potential cryptic species, misidentified as *E. complanata*, and evidence for a cryptic phylogenetic species within *L. radiata radiata* both appeared in our collection. Together, our results validate the use of hemolymph extraction, followed by PCR-RFLP assays and targeted DNA sequencing, as a rapid, inexpensive, and reliable identification method for field biologists at NCDOT and other agencies who routinely survey NC freshwater mussel populations.

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I. Introduction

North America boasts the highest diversity of freshwater mussel species anywhere. Almost three-quarters of these species, however, are at risk of extinction (Stein and Chipley 1996), and like many freshwater organisms, are particularly threatened by construction of roads, bridges, and residential and commercial structures. Of the 60 freshwater mussel species reported from North Carolina waters, 35 are considered to endangered, or are species of special concern to state and federal agencies (NC Mussel Atlas: http://www.ncwildlife.org/fs_index_07_conservation.htm: Alderman et al. 2007). State and federal regulators, therefore, are increasingly asking agencies like the NC Department of Transportation to document whether a threatened or endangered freshwater mussel exists at a proposed construction site. Since the presence of an endangered species can delay or greatly increase the cost of a construction project, methods for identifying and distinguishing freshwater mussel species at field sites are therefore in great need.

Biologists have generally relied upon physical characteristics to delineate species of freshwater mussels, but this approach is notoriously difficult to implement and may be unreliable for distinguishing some taxa. Even distantly related species can show very similar external characteristics, and shell traits are known to vary in response to environmental conditions (Baker et al. 2004). Molecular genetic methods provide a powerful alternative for identification. Sequences of both mitochondrial and nuclear DNA regions have been used very effectively to distinguish species and evaluate the genetic relationships among populations of freshwater mussels (*e.g.* King et al. 1999; Roe et al. 2001; Serb et al. 2003; Hughes et al 2004). There are, however, logistical problems associated with genetic methods. First, mortality from tissue sampling by biopsy or by collecting whole animals prevents biologists from sampling populations at risk and hinders development of methods for distinguishing endangered from non-endangered populations. Secondly, methods that rely upon DNA sequencing require expensive equipment and materials and trained personnel that limits their applicability by NCDOT and other agencies. This project was designed to test methods to minimize these logistical limitations on the implementation of genetic methods for identifying freshwater mussel species in North Carolina waters.

Recent work with captive freshwater mussel populations (Gustafson et al. 2005) showed that removal of 0.5cc of hemolymph from the adductor mussel of *Elliptio complanata* did not result in high levels of mortality; survival was 90% after 13 weeks. Similar work with marine mussels showed that mortality of animals from which hemolymph was obtained was not significantly different from controls up to a year after sampling (Yanick and Heath 2000). Raley et al. (2006) reported that hemolymph removal from *Elliptio complanata* is nonlethal if sampling does not occur more than once a month. The latter studies, moreover, reported that the extracted hemolymph performed similarly to solid tissue samples with respect to yielding DNA suitable for genetic analysis (Yanick and

Heath 2000; Raley et al. 2006). These results show that drawing hemolymph from mussels produces little or no mortality, and is therefore a viable method for non-lethal sampling that deserves evaluation in natural populations of endangered or threatened species

This project field-tested hemolymph collection as a method for the non-lethal collection of tissues for DNA extraction and genetic identification of freshwater mussels. We based our work in Lake Waccamaw. This is the largest Carolina Bay in North Carolina, from which seventeen species of freshwater mussels been reported to occur, either at present or historically (Bogan, 2002). Not all of these species are abundant, however, so we focused our work on two described endemics, the state endangered species *Elliptio waccamawensis* and the state threatened species *Lampsilis fullerkati*, both of which (particularly the former) maintain large populations in Lake Waccamaw, but are found nowhere else. Like several other freshwater mussels that are threatened or even more critically endangered, these two taxa are genetically distinct but difficult to distinguish morphologically. Hence they serve as valuable test case for the application of non-lethal, rapid genetic identification methods to freshwater mussels, but on a fauna that is accessible and amenable to field experimentation, locally abundant so that it can withstand minor impacts imposed by sampling efforts, yet still of conservation relevance.

Our project included three major components. First, we assessed the effects of hemolymph sampling on mussel growth and survival under conditions in their native environment, through field experiments conducted in Lake Waccamaw. Second, we developed polymerase chain reaction (PCR) assays of mitochondrial DNA (mtDNA), followed with digestion by restriction enzymes that reveal species-diagnostic DNA restriction fragment length polymorphisms (RFLP). These assays unambiguously distinguish between the two endemics, and as we demonstrated in field trials at two locations in Lake Waccamaw, were effective in detecting the very few cases where experienced biologists working with us had misidentified specimens showing ambiguous morphological features. Thirdly, to evaluate the taxonomic status of the two Lake Waccamaw endemic species, we collected several individuals of candidate sister taxa from throughout nearby rivers in the Lumber River and Yadkin-Pee Dee River Basins of southeastern North Carolina, and performed phylogenetic analysis of sequences from two mtDNA regions. Based on these analyses, we have produced a set of reliable, rapid and relatively inexpensive protocols for genetic identification of the Lake Waccamaw species and other related taxa in southeastern North Carolina. Verification of the identity and relationships between these taxa is being carried out by ongoing work on the systematics of *Elliptio* and *Lampsilis* in our region by biologists at the NC Museum of Natural Sciences and throughout the Southeast. Pending the completion of their work and any refinements deemed necessary as a result, our methods could be readily implemented by biologists at NCDOT and other agencies charged with surveying, monitoring and restoring freshwater mussel populations in our state.

II. Methods

A. Field assessments of growth and survival

i. Experimental design

Two enclosures were constructed in Lake Waccamaw (Columbus County, NC, map: Fig. 1) in April 2004. The enclosures were placed about 300 meters offshore (Fig 1, site 1) in Lake Waccamaw State Park in about 1.5 meters water depth. Each enclosure was constructed of wire fence material onto which plastic 3/4" mesh was attached and the enclosures were anchored into the sand with re-bar stakes. Each enclosed approximately 10 m^2 area of the sand lake bottom and stood about 25 cm above the sand. All freshwater mussels were cleared from the enclosures prior to stocking them with experimental animals. To do so, we collected all of the mussels on and under the sand surface and placed them into mesh bags. We found that a few animals missed on our first subsurface search would later appear on the sand surface; *Leptodea ochracea,* in particular, was easy to miss in this way because of an apparently greater tendency to burrow than the other 2 species. After 30 minutes and again after 1 hour, we repeated the clearing procedure until no further animals were encountered. This same search process was used on subsequent dates on which we recollected and measured the test animals within the enclosures.

Individuals of the three most abundant freshwater mussel species in the Lake: *E. waccamawensis*, *L. fullerkati*, and *L. ochracea*, were selected as test species. The species of *Elliptio* and *Lampsilis* have been described as endemics in the lake (Johnson 1970, 1984). *E. waccamawensis* occurs at a density high enough such that all of the test individuals used in an enclosure were collected from within that enclosed area while it was being cleared of animals. Both *L. fullerkati* and *L. ochracea* were considerably less abundant, so test individuals of these species had to be collected from a broader area (about 200 – 300 m^2 surrounding site 1, Fig. 1) after extensive searching. Mussels were identified to species based on morphological criteria by Dr. Ryan Heise and Mr. Rob Nichols from North Carolina Wildlife Resources Commission (NCWRC).

Into each enclosure, we placed 20 *E. waccamawensis*, 16 *L. fullerkati*, and 20 *L. ochracea*. Preliminary data revealed that the average density in the Lake at the depth where we were establishing the enclosures was ~5/m^2 (test transects were 50 m long X 2 m wide and averaged 500 animals per transect). Each species however, was not equally represented and thus the experimental densities were 0.5, 5, and 30 times the natural abundance for *Elliptio*, *Leptodea* and *Lampsilis*, respectively. Experimental densities were elevated relative to natural abundances in the two less common species in order to provide sufficient sample sizes for reliable mortality and growth estimates. We attempted to equalize sample sizes as much as possible, but after a half-day's searching, we collected only 32 *Lampsilis*, and so we used all of these as test animals and equalized the sample sizes of the other 2 species at 20 each.

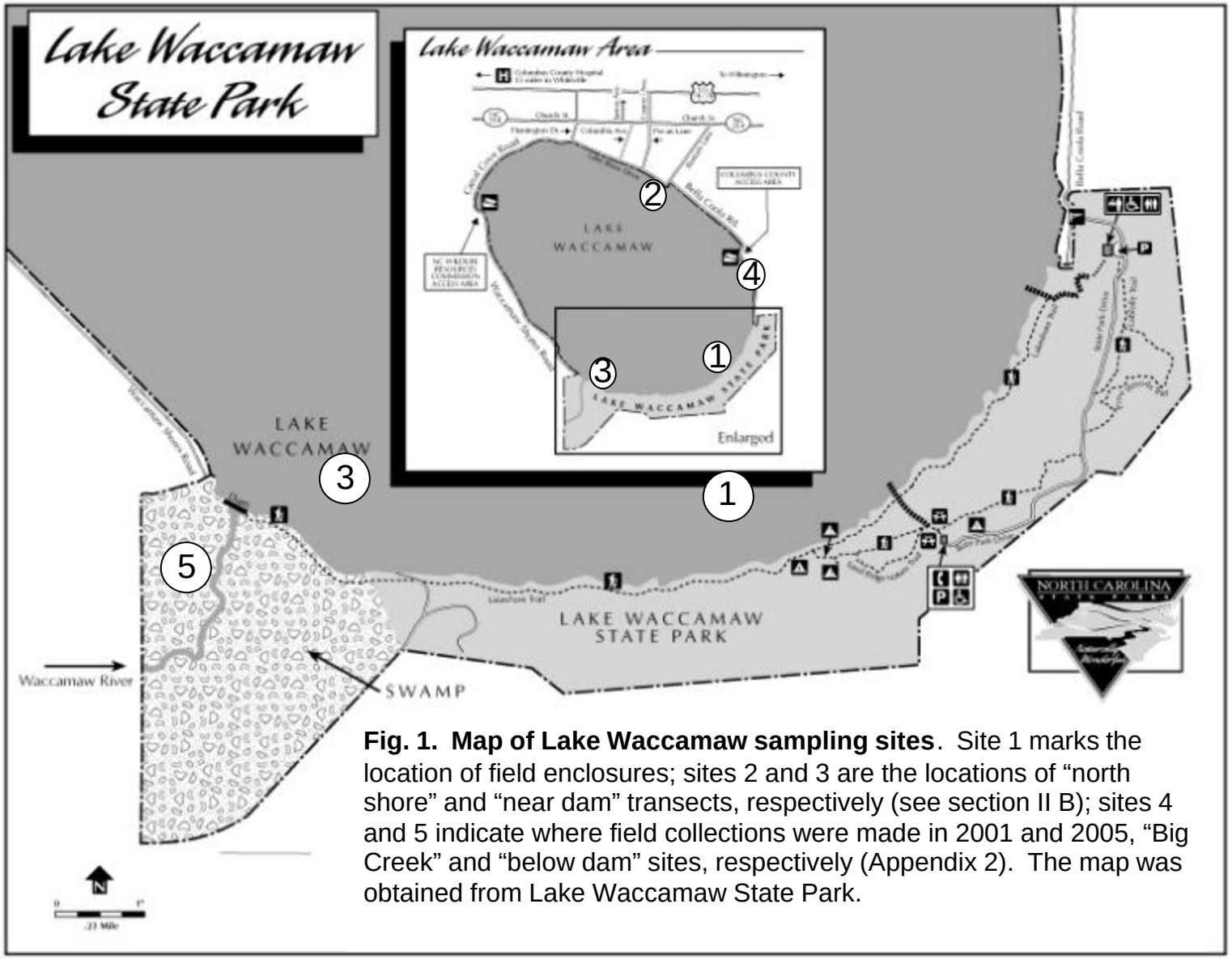


Fig. 1. Map of Lake Waccamaw sampling sites. Site 1 marks the location of field enclosures; sites 2 and 3 are the locations of "north shore" and "near dam" transects, respectively (see section II B); sites 4 and 5 indicate where field collections were made in 2001 and 2005, "Big Creek" and "below dam" sites, respectively (Appendix 2). The map was obtained from Lake Waccamaw State Park.

From one-half of each of these sets of animals, 20-100 µl of hemolymph was extracted from the anterior adductor muscle using a 1 ml sterile syringe fitted with a 27G1/2 needle and the hemolymph samples were immediately placed on ice. The animals were then measured with electronic calipers and placed back into the enclosure. The remaining one-half of the animals were not extracted but were otherwise handled similarly, and their survivorship and growth was monitored as control animals. All animals were tagged with "bee-tags" (The Bee Works, Orillia, ON Canada) so that they could be individually identified.

ii. Growth and survival

The growth and survival of all mussels was assessed at 2, 4 and 8 weeks post-hemolymph removal. At each sampling date, all mussels inside of the enclosures were removed (as described above) and enumerated. Each individual was re-measured, inspected to make sure they were alive and not moribund, and then were returned to the enclosures. Analysis of variance was used to test for differences in growth between the treatments (hemolymph-sampled and controls) for each species.

At the end of the experiment, all animals were collected, transported on ice to UNCW, and are presently stored frozen at -40ºC. The number of these whole-animal samples that are contained in our collection is provided in Appendix 2. At the conclusion of this study and of the completion of the thesis of Ms. Kristine Sommer, the graduate student whose work was funded by this grant, shells, soft tissues, and tissue extracts of all of the specimens will be deposited at the North Carolina Museum of Natural Sciences in Raleigh. We will also provide a DNA sequence of no fewer than 2 of specimens, cross-reference to voucher index number, and all DNA sequences from this study wil be deposited on GenBank. We anticipate that the specimens will be transferred in May 2007.

iii. Confirmation of morphological identifications

Using the methods outlined below (B. Genetic Methods), mussels evaluated for growth and survival were analyzed to confirm species identity. For the control animals, extracts were generated from mantle tissue samples collected from the frozen specimens.

iv. Genetic methods

a. <u>DNA extraction</u>

DNA was extracted from two tissues for genetic analysis. We used 2-3 mm^3 pieces of mantle tissue from frozen specimens and/or the 20-100 µL of hemolymph and applied a modification of the PureGene DNA extraction kit protocol (Gentra Systems, Minneapolis MN). The modifications to the manufacturer's methods are as follows and generally were made to account for the small sample volumes used in our study. A total of 200 µL of cell lysis solution and 1.5 µL of Proteinase K solution were combined with 10 to 50 µL of hemolymph (or the tissue sample) and incubated overnight at 55ºC. Proteins, RNAs, and other cellular debris were separated from DNA by adding 70 µL of protein precipitation solution, vortexing, chilling for 5 min at 4ºC and centrifuging at 17,000 G for 5 minutes at 4ºC. To precipitate DNA from the supernatant fraction, we

added 200 µL 100% isopropanol, mixed gently, and centrifuged at 17,000 G for 5 minutes at 4ºC. The DNA pellet was then washed with 100% isopropanol, followed by 70% ethanol. The ethanol was decanted and the pellets were dried under vacuum, and were resuspended in 35 µL Polymerase Chain Reaction (PCR)-grade water.

A StrataPrep PCR Purification Kit (Stratagene, La Jolla CA) was then used to further purify the DNA extracts, following the kit protocol. In our initial work, we found this additional purification to greatly improve our PCR amplification success from hemolymph DNA extracts. The eluted DNA (from the Stratagene procedure) was then vacuum-dried, resuspended in 10-15 µl of PCR-grade water and stored at -20ºC until analysis.

b. PCR amplification

Three gene regions were PCR-amplified using a PTC-100 Thermal Cycler (MJ Research Inc., Waltham MA). The cytochrome oxidase I (COI) and 16s ribosomal DNA regions are from the mitochondrial genome; the internal transcribed spacer (ITS) region is from the nuclear genome. The reactions were carried out in 25 µl volumes containing 10 X PCR buffer, dNTPs (2µM each), forward and reverse primers (10µM each), Taq polymerase (ABI, Valencia CA), and PCR-grade water. The primers and cycling conditions used to amplify these regions are shown in Table 1.

c. DNA Sequencing

Primers and salts were removed from the amplified segments of DNA using the StrataPrep PCR Purification Kit (Stratagene, La Jolla CA). PCR products were sequenced from both the forward and reverse primers using the Applied Biosystems (ABI, Foster City CA) Big Dye Terminators Kit Version 3.1 and the ABI 3100 Genetic Analyzer. Sequences were edited using Sequencher (Gene Codes Corporation, Ann Arbor MI), aligned using Clustal X (Thompson et al. 1994) and the alignment was imported into MacClade 4.0 (Sinauer Associates, Sunderland MA) for final editing.

d. Design of restriction fragment length polymorphism assays

Sequence data for the 16s rDNA region was evaluated for species specific differences between *L. fullerkati* and *E. waccamawensis*. Three restriction enzymes were selected as candidates for diagnostic assays and tested using mussels collected as part of the field trials. Purified 16s PCR products from were digested with *Hin*f I, *Ava* II, and *Hind* III restriction endonucleases [New England BioLabs (NEB), Beverly MA]. All digests were in 20 µL volume reactions, containing 10 µL of the following cocktails and 10 µL of cleaned PCR Products. *Ava* II cocktails contained 8.2 µL PCR H_2O, 1.0 µL 10 x NEBuffer #4, and 0.8 µL *Ava* II (10 units/µL). *Hin*f I cocktails contain 8.2 µL PCR H_2O, 1.0 µL 10 x NEBuffer #2, and 0.8 µL Hinf I (10 units/µL). *Hind* III cocktails contained 8.0 µL PCR H_2O, 1.0 µL 10x NEBuffer #2, and 1.0 µL *Hind* III (20 units/µL).

Table 1: PCR primers (5´ to 3´), cycling conditions, and product sizes. The sequences for the primers LCO1490 and HCO2198 were reported Folmer et al. 1994, primer White 18s was reported in White et al., 1994 and 28sMulvD3 was reported in Mulvey et al., 1998

Gene Region	Primers	Annealing Temp	Product Size
16s rDNA	16SUN693F: AGATAATGCCTGCCCAGTG 16SUN1178R: CGGTCTTAACTCAGCTCGTGTA	50ºC	487bp
COI	LCO1490:GGTCAACAAATCATAAAGATATTGG HCO2198:TAAACTTCAGGGTGACCAAAAAATCA LCO1490D:GNTCNACNAATCATAARGATATTGG HCO2198D:TAAACYTCAGGRTGNCCAAAAAATCA	45ºC	750bp
ITS	White18S: TAACAAGGTTTCCGTAGGTG 28SMulvD3: CCTTCTCAGGCATAGTTCACCATC	50ºC	3kb

Table 2: Predicted fragment sizes for the diagnostic PCR-RFLP of 16s rDNA in Lake Waccamaw endemic species based on sequence data

Enzyme	Species	Number of DNA fragments	Fragment sizes (bp)	
Ava II	*E. waccamawensis*	1	440	
	L. fullerkati	2	280	160
*Hin*f I	*E. waccamawensis*	2	238	202
	L. fullerkati	1	440	
Hind III	*E. waccamawensis*	2	120	320
	L. fullerkati	1	440	

The samples were incubated at 37ºC for at least 16 hours, then the digested products were loaded onto 1.8% NuSieve 3:1 agarose gels (Cambrex Bio Science, Inc., Rockland ME) containing ethidium bromide (0.25 µg/ml). The banding patterns were visualized under UV light. Table 2 shows the predicted fragment sizes.

B. Field trial of genetic identification methods in Lake Waccamaw

In August 2004, at two locations in Lake Waccamaw were surveyed using the methods of hemolymph extraction and PCR-RFLP to check morphological identifications of mussels. Our goal was not to attempt a census of mussel diversity in the Lake, a goal clearly beyond the reach of our limited spatial sampling and outside the scope of this project. Rather, we view this as a first field-based evaluation of an application of our methods. Lake Waccamaw is an ideal site in which to carry out such and evaluation. For one, animals are abundant and easy to access, and any mortality we may have inflicted would have little impact on the large Lake populations. And secondly, the numerically dominant Lake species, *E. waccamawensis*, co-occurs with a much less abundant, genetically very distinct species (*L. fullerkati*) that is nevertheless morphologically difficult to distinguish. Hence this field trial could serve as a model for

other cases that may confront field biologists surveying sites where for example a morphologically cryptic, threatened species co-occurs with a more abundant species.

We used belt transects deployed at two nearshore sites that appeared ecologically distinct. Transects measured 30 m long and all mussels within 1m to each side of the line were collected, identified by NCDOT personnel and each species was enumerated. Mussels were photographed, and hemolymph was sampled from 8-10 individuals of each species (when numbers allowed).

The north shore site (N 34.3069º W 78.5012º) has deeper water near the shore, so we selected an area with similar water depth (1.5 –2 m) as the site we had sampled near the State Park. The substrate at the north shore site has considerably more organic material and much more abundant emergent aquatic vegetation (*Nuphar* sp. and *Panicum* sp.) than at the State Park site where the enclosures were placed. A single transect at the north shore site yielded approximately 450 mussels. A second site was located just east of the small dam at the head of the Waccamaw River (N 34.2610º W 78.5180º). Dense growths of maidencane (*Panicum* spp.) and bald cypress (*Taxodium ascendens*) near the shore characterize this site. At this site, we collected 130-300 mussels from 2 X 60 m^2 belt transects. Hemolymph samples were processed as previously described and subjected to analysis by the PCR-RFLP methods described above.

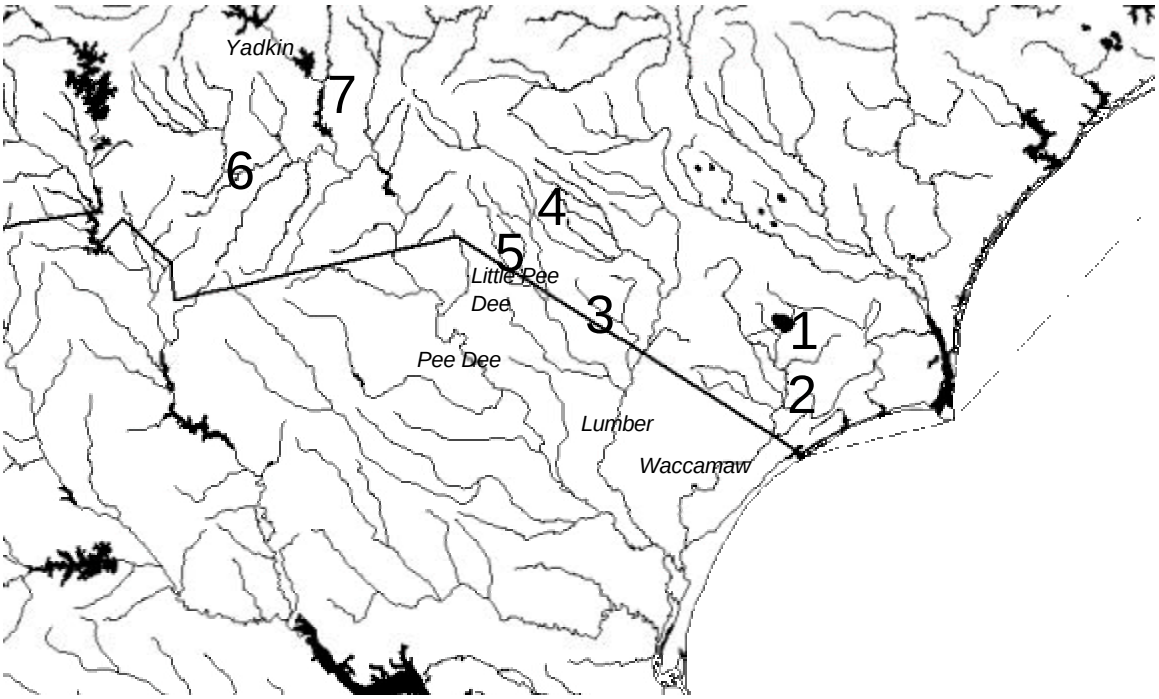
C. Phylogenetic analysis of Lake Waccamaw endemic freshwater mussels and candidate sister species in southeastern North Carolina rivers

i. Sampling and DNA sequencing

Hemolymph was sampled from a total of 387 specimens representing 18 putative species from 12 different geographic populations. The motivation for this survey was two alternative hypothesis: one, that the Lake Waccamaw species were genetically indistinguishable from a geographically more widespread species, and two, that the Lake Waccamaw species were distinct from, but sister to, a species present in nearby rivers. Since Lake Waccamaw is essentially the head of the Waccamaw River, and since the Waccamaw River is essentially an arm of the Great Pee Dee Drainage, we addressed these two hypotheses by concentrating our sampling efforts in the Waccamaw River and other rivers in the Lumber and Yadkin-Pee Dee Basins (Fig. 2). In addition, a single specimen of *Lampsilis* was obtained from both the Tar and Flat Rivers due to its unknown affinities yet suspected similarity to *L radiata radiata* (see Appendix 2 for a list of taxa and sampling locations). Vouchers of selected taxa were collected for a total of 166 individuals (Appendix 2), and each of these will be deposited at the North Carolina Museum of Natural Sciences in May-June 2007. DNA extraction, PCR, and DNA sequencing was performed as described above for two mtDNA regions (16s rDNA and COI).

Sequences of both regions from six additional taxa were obtained from GenBank. We used the advice of Dr. Bogan and the study of Campbell et al. (2005) to guide selection of these species. Our goal was to more clearly determine the taxonomic

affinities of the taxa we collected, and to recheck all identifications, since these relied entirely upon inspection of shell characters by NCWRC biologists. More specifically, we chose sequences from type specimens (Campbell et al. 2005) for the genera *Elliptio, Lampsilis, Leptodea,* the three genera most common in Lake Waccamaw. Additionally, we chose type specimens of *Pleurobema* and *Fusonaia* as outgroups to *Elliptio* within the tribe Pleurobemini. Our intent was not to address the monophyly of this or any other higher taxon of unionids, which is of course outside of the scope of this study, but was simply to check whether our field identified animals grouped in a manner consistent with published phylogenies. Finally, we included *Uniomerus declivus* as an outgroup to all species in the trees, and *Elliptio dilatata* just for fun. Identity of these downloaded sequences and GenBank accession numbers are provided in Appendix 3.



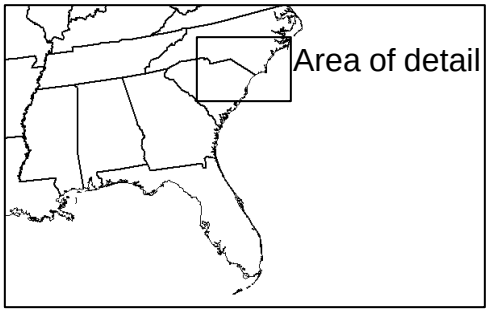


Fig. 3. Locations of populations sampled in the Lumber and Yadkin-Pee Dee River Basins. Sites in the Lumber River Basin are: (1) Lake Waccamaw, Columbus County; (2) Waccamaw River, Columbus Co; (3) Hog Swamp, Lumber River, Robeson Co.; (4) Richland Swamp and Hwy 71 Bridge, Lumber River, Robeson and Scotland Cos.; (5) Shoe Heel Creek, Little Pee Dee River, Robeson Co.; and in the Yadkin-Pee Dee River Basin: (6) Lick Creek, Pee Dee River, Anson Co.; (7) Morrow Mnt. State Park, Yadkin-Pee Dee River, Stanly Co. Major rivers are labeled in italics. Map of river drainages was obtained from the National Atlas of the U.S., Dept. of Interior.

ii. Methods of phylogenetic analysis

For the CO I data, neighbor-joining (NJ: Saitou & Nei 1987) phylogenies were constructed using PAUP 4.0b10 (Swofford 2002). In our ongoing work, we are conducting both NJ and Bayesian analysis, the latter using MrBayes 3.1 (Ronquist et al. 2005), but we present only NJ trees here. This is due to the preliminary nature of this analysis, which at present lacks sequences of type specimens provided to us by Dr. Bogan, which we are now sequencing and adding to the alignment. MrBayes analyses requires considerable computation time, so we chose to delay this until the entire alignment was complete. Inclusion of these new sequences and the more comprehensive analysis forms a follow-up to this report and is part of the thesis of Kristine Sommer.

Similarly, due to our incomplete taxon sampling, at present we are using a simplified model of molecular evolution, the model of Tamura and Nei (1993). We have found that best-fitting models of molecular evolution, obtained from Modeltest 3.06 (Posada & Crandall 1998), are similar to the model employed, but the exact parameters selected (particularly with respect to rate heterogeneity) are sensitive to taxon sampling, so we decided to wait until the full alignment is complete for a final evaluation of the appropriate model. Throughout this report, we define *clade* as a monophyletic group of closely related sequences, including the ancestor and all of the descendents of that ancestral sequence. Support for clades in the NJ tree was derived from 1000 NJ bootstrap replications. We have found that support for shallow clades (including those containg *Elliptio waccamawensis)* is sensitive to the methods of phylogenetic analysis and molecular evolutionary model, so the precise values for support should be viewed with some caution. Nevertheless, we restrict most of our discussion to clades with high bootstrap support.

III. RESULTS

A. Field assessments of growth and survival

The fraction of individuals recovered was very high and not different between the two enclosures, so the values for both enclosures were pooled (Table 3). Only a single animal was found dead at the end of the experiment and it was a control animal. A total of 8 individuals were not recovered at the end of the experiment; 5 of these were control and 3 were hemolymph-extracted animals. The lost animals either escaped from the enclosures, most likely by burrowing underneath the fence or (less likely) were so deeply burrowed that they were missed during sampling. An observation consistent with either explanation is that 7 of the 8 individuals lost were *L. ochracea*, which is the species that we observed to burrow rapidly, and deeper than the other species.

Table 3. Growth and survivorship of hemolymph extracted and control animals over 8 weeks in Lake Waccamaw field enclosures. *Two of the individuals placed in this *Lampsilis* control group have subsequently been identified as *E. waccamawensis*. (see "Genetic identification" section below).

Genus	Treatment	Number of mussels Week 0	Number of mussels Week 8	Mean ? shell length (mm)	Standard Error of the Mean
Elliptio	Control	22	21	0.62	0.11
	Extracted	20	20	0.57	0.08
*Lampsilis**	Control	14	13	0.45	0.08
	Extracted	16	16	0.42	0.08
Leptodea	Control	20	16	3.20	0.16
	Extracted	20	17	3.03	0.17
Total	Control	56	50		
	Extracted	56	53		

From these trials, there was no indication that the removal of hemolymph led to increased mortality among the experimental animals. Further, the data on growth, as measured as the increase (?) in maximum shell length (in mm) after 8 weeks suggests few significant effects of the hemolymph removal. Both control and experimental groups showed an increase in size in all 3 species. *L. ochracea* was the fastest growing of the species, growing at a rate that was 5.1 and 7.2 times faster than *E. waccamawensis* and *L. fullerkati*, respectively.

Table 4. 2-Way ANOVA on species differences in growth and the effect of hemolymph extraction on growth in field enclosures. df = degrees of freedom; ***P < 0.001; ns = not significant (P > 0.05

Factor	df	Sum of squares	Mean square	F
Species	2	149.2	74.60	299.1***
Hemolymph Extraction	1	0.192	0.192	0.772 ns
Interaction (Species X Extraction)	2	0.082	0.041	0.165 ns
Error	96	23.94	0.249	

A 2-way Analysis of Variance (ANOVA: Table 4) showed that growth rate differences between species were highly significant, and post-hoc analysis (Fisher's PLSD) showed a highly significant difference between *L. ochracea* and both of the other two species (P < 0.001 for both comparisons), but no significant difference between *E. waccamawensis* and *L. fullerkati* (P = 0.189). There was a 5-8% diminution in growth in the hemolymph-extracted group of animals compared to controls in all 3 species, but neither the main effect nor the interaction between extraction treatment and species were significant by 2-way ANOVA (Table 4). The raw data are provided in Appendix 1.

ii. Confirmation of morphological identifications genetic identifications using PCR-RFLP

Individuals of both *E. waccamawensis* and *L. fullerkati* were used to confirm that hemolymph can be used instead of tissue as a source of DNA. We compared success rate of 16s rDNA PCR amplification from hemolymph DNA extracts relative to that of mantle tissue DNA extracts and then compared the DNA sequences generated from the 2 sets of amplicons. The results showed that success rate from hemolymph was ≥ 90%, but lower than success rate from mantle, which was 100% (Table 4). DNA sequences from hemolymph DNAs were identical to those from mantle tissue. Raley et al. (2006) have recently reported comparable results, using hemolymph as a source of DNA for sequencing mtDNA regions from *Elliptio complanata.* While they do not report % PCR success rate, they do note identity or near identity of DNA sequences derived from hemolymph to those derived from mantle tissue, across a set of *E. complanata*. In So far, we have encountered no discepancies between hemolymph derived DNA sequences and those derived from body tissues of voucher specimens, across several taxa from our regional survey (not shown). Together, these data indicate that hemolymph is likely to generally be a reliable source of DNA for genetic analysis in freshwater mussels.

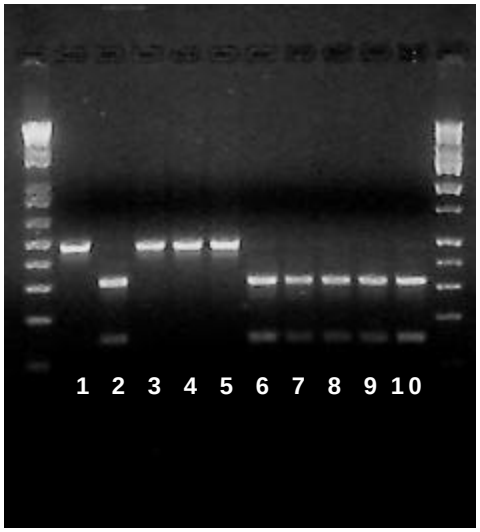
Table 4. Success rate of 16s rDNA PCR amplification from mantle tissue and hemolymph based DNA extracts. Values are number of products/number of attempts.

Species	Mantle tissue	Hemolymph
E. waccamawensis	24/24 (100%)	18/20(90%)
L. fullerkati	20/20 (100%)	15/16 (94%)

Identification of all mussels used in the growth and survival studies were genetically typed using PCR-RFLP assays (see Methods). PCR-RFLP assay gels using 2 informative restriction enzymes can be seen in Figure 3 and conform to expected patterns reported in Table 2. *Hin*f I and *Hind* III each cut 16s rDNA PCR products from *E. waccamawensis* into two fragments, but did not cut products from *L. fullerkati*. Conversely, *Ava* II cut the *L. fullerkati* 16s amplicons into 2 fragments, but did not cut *E. waccamawensis* products. On both the *Hin*f I and *Ava* II gels, lane 2 shows the fragment patterns produced from an individual whose RFLP phenotype with both enzymes did not agree with its morphological identification as *L. fullerkati*. Subsequent DNA sequence analysis of this individual confirmed that it was *E. waccamawensis.*

Each time we encountered a discrepancy between RFLP and morphological identification, we sequenced the DNA of the PCR product from the individual in question. Morphological misidentification of *E. waccamawensis* as *L. fullerkati* occurred in a total of 2 individuals used in the enclosure experiment (Table 5). In each case, the misidentification was detected using each of the 3 restriction enzyme assays, and in each case, the individual was a control animal.

Hinf I



Ava II

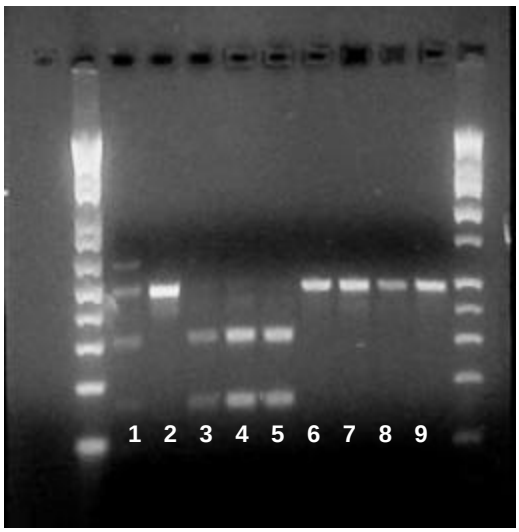


Figure 3. RFLP analysis of 16s PCR products. Agarose gels showing the products of *Hinf* I and *Ava* II digests. Outside lanes on each gel contain DNA size ladders. In both gels, lanes 1-5 contain amplicons from the same 5 *L. fullerkati* individuals; lanes 6-9 contain amplicons from the same 4 *E. waccamawensis* individuals; lane 10 on the *Hinf* I gel is an additional *E. waccamawensis* individual. All animals were morphologically identified to species. According to the results, the animal in lane 2 was morphologically misidentified as *L. fullerkati*, and this conclusion was confirmed by DNA sequencing. On the *Ava* II gel, lane 1 is an example of an "ambiguity" due to poor PCR yield and incomplete digestion. Upon DNA sequencing, this animal was confirmed to be *E. waccamawensis*

Sequence analysis was also utilized when the RFLP phenotype was ambiguous (Table 5, Fig. 3) or unusual. In 2 cases, digestion with the enzyme *Hin*f I, yielded a clear RFLP result that conflicted with morphological identification, but upon sequencing, we found that the 16s sequence agreed with the morphological identification of *E. waccamawensis*. Inspection of these sequences revealed the presence of restriction site variant that in *E. waccamawensis* that occurred at a frequency of ~5% (2/39). Consequently, we discontinued use of *Hin*f I for subsequent diagnostic work on these species. Extensive sampling with both *Ava* II and *Hind* III (Tables 5 and 7) has revealed no polymorphism, so both of these enzymes appear to be diagnostic for these morphologically very similar species.

Table 5. Results of 16s PCR-RFLP identifications of animals used in Lake Waccamaw field enclosures.

Enzyme	Morphological species	Number typed	RFLP & morphology consistent	RFLP & morphology inconsistent	RFLP ambiguous	RFLP site variant
Ava II	*E. waccamawensis*	39	38	0	1	0
	L. fullerkati	30	28	2	0	0
Hinf I	*E. waccamawensis*	39	36	0	1	2
	L.fullerkati	30	27	2	1	0
Hind III	*E. waccamawensis*	39	37	0	2	0
	L. fullerkati	30	28	2	0	0

B. Field trial of genetic identification methods in Lake Waccamaw

Densities of animals were very high at the north shore site (mean of 7.5/m^2) but densities were lower (3.7/m^2) and distributions more patchy at the dam site (Table 6).

Table 6. Freshwater mussel belt transect surveys in Lake Waccamaw

Site	Transect	Species	N	%	Number sampled (hemolymph)
North shore	1	*Leptodea ochracea*	41	9.1	10
		Elliptio waccamawensis	399	89.0	10
		Elliptio folliculata	1	0.2	1
		Lampsilis fullerkati	6	1.3	6
		Unknown	1	0.2	1
		Total	448		
North shore	2	*Leptodea ochracea*	55	12.0	10
		Elliptio waccamawensis	400	87.3	10
		Lampsilis fullerkati	3	0.6	3
		Total	458		
Near dam	1	*Leptodea ochracea*	8	6.0	8
		Elliptio waccamawensis	125	93.3	5
		Lampsilis fullerkati	1	0.7	1
		Total	134		
Near dam	2	*Leptodea ochracea*	20	6.4	2
		Elliptio waccamawensis	286	92.0	5
		Elliptio folliculata	2	0.6	1
		Lampsilis fullerkati	3	1.0	3
		Total	311		

Percent composition was very similar, with rank abundances being *E. waccamawensis* (90%), *Leptodea ochracea* (6-12%), and *Lampsilis fullerkati* (~1%), with an occasional *E. folliculata* encountered. PCR-RFLP identifications were consistent with morphological identifications in 42/43 cases (Table 7). Only one *Hind* III ambiguity was found for a single *Lampsilis fullerkati*, and its correct morphological ID was confirmed through sequencing. A single morphological misidentification was discovered in the case of 1 putative *Lampsilis fullerkati*. Both RFLP assays and DNA sequencing confirmed that this individual was actually *E. waccamawensis*. This result again demonstrates the power of PCR-RFLP for checking and confirming identifications of morphologically conservative freshwater mussels in field surveys.

Table 7. PCR-RFLP of animals from the belt surveys

Enzyme	Morphological species	Number typed	RFLP & morphology consistent	RFLP & morphology inconsistent	RFLP ambiguous	RFLP site variants
Ava II	*E. waccamawensis*	30	30	0	0	0
	Lampsilis fullerkati	13	12			
Hind III	*E. waccamawensis*	30	30	0	0	0
	Lampsilis fullerkati	13	11	1	1	0

Figure 4b. Bootstrap consensus of 16s sequence data. See Fig. 4a legend for details.

C. Phylogenetic analysis of Lake Waccamaw endemic freshwater mussels and related species in southeastern North Carolina Rivers

Our 16s tree (Fig. 4a, see Table 8 for descriptions of shared haplotypes) showed strong support for monophyly of the tribe Pleuroblemini, containing *Elliptio* and the outgroup genera *Pleurobema* and *Fusconaia*. This lends support to genus level identification of most *Elliptio* from our field census (see below). Within the Pleurobemini, the tree showed strong support for monophyly of *E. fisheriana*, and also showed strong support for a clade containing the lance-like species *E. folliculata*, *E. producta*, and the "Pee Dee Lance" (*E. angustata*), but none of these species was individually monophyletic. Most importantly for our study, there was a loose grouping of *E. complanata*, *E. congaraea*, *E. icterina*, *E. waccamawensis*, and several unidentified *Elliptio* into phylogroup **A**, but none of these species was monophyletic. So in the 16s data set, data set, *E. waccamawensis* was not supported as a distinct monophyletic species whose lineage is endemic to Lake Waccamaw. In fact, several individuals (Fig 4a, Table 8) shared identical haplotypes with other *Elliptio.*

One striking finding in the 16s data set concerns the affinities of 7 individuals collected from the Pee Dee and Lumber Rivers —5 of which were

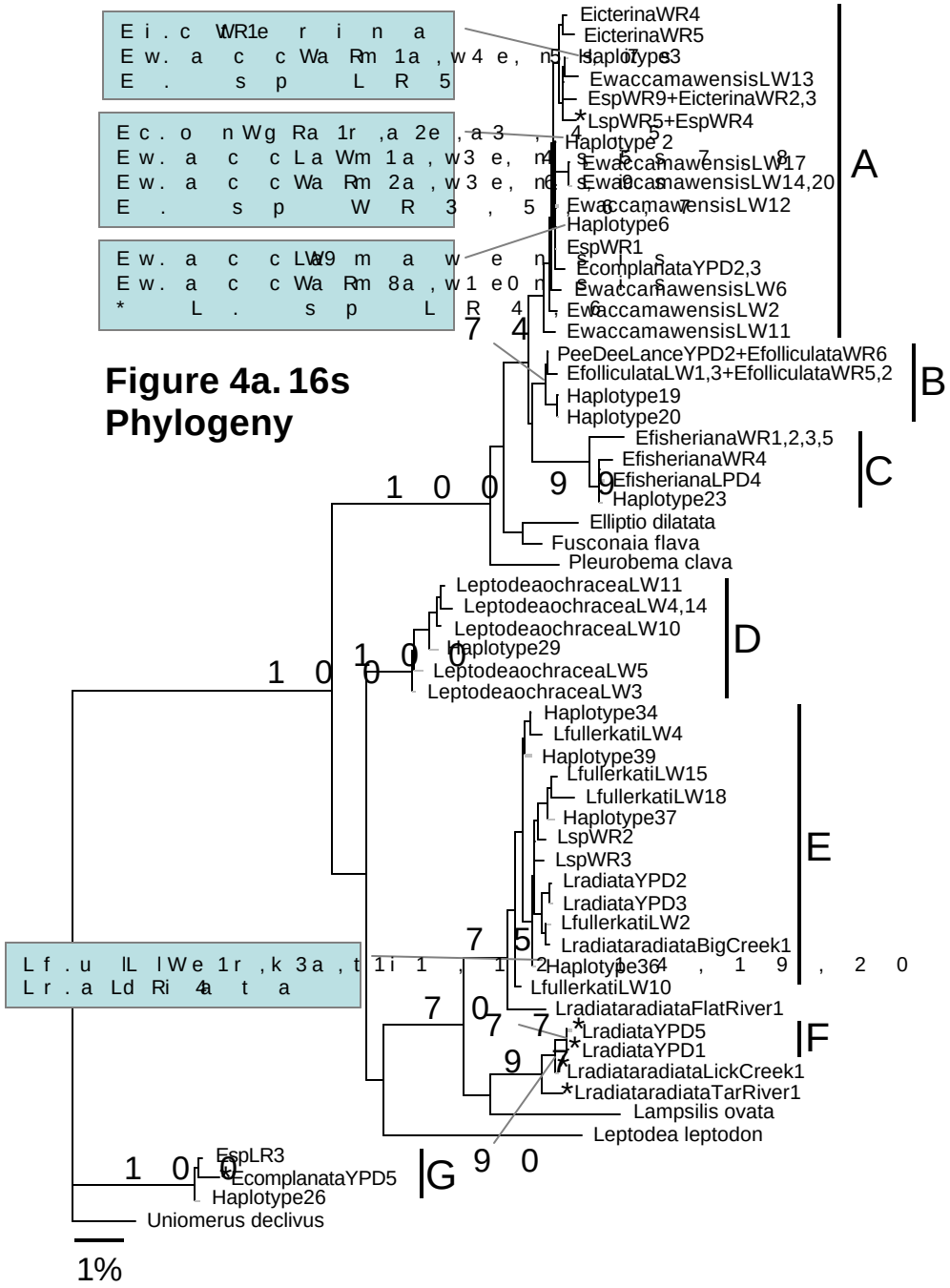


Figure 4a. 16s Phylogeny

Figure 4a. Neighbor-joining phylogram of 16s sequence data. Numbers on branches are bootstrap support (1000 NJ replications). Values less than 70 are not shown, and some values for subclades are not shown; see Fig. 4b for all values. Callout boxes show individuals that share identical, numbered haplotypes. Taxa are labeled with morphological species (asterisks mark likely misidentifications), followed by location abbreviation (LW=Lake Waccamaw, WR= Waccamaw River, YPD= Yadkin-Pee Dee River, LPD=Little Pee Dee River), followed by individual ID #. Commas separate individuals from the same location that share haplotypes. Vertical bars with bolded letters designate well supported clades, or phylogroups discussed in the text.

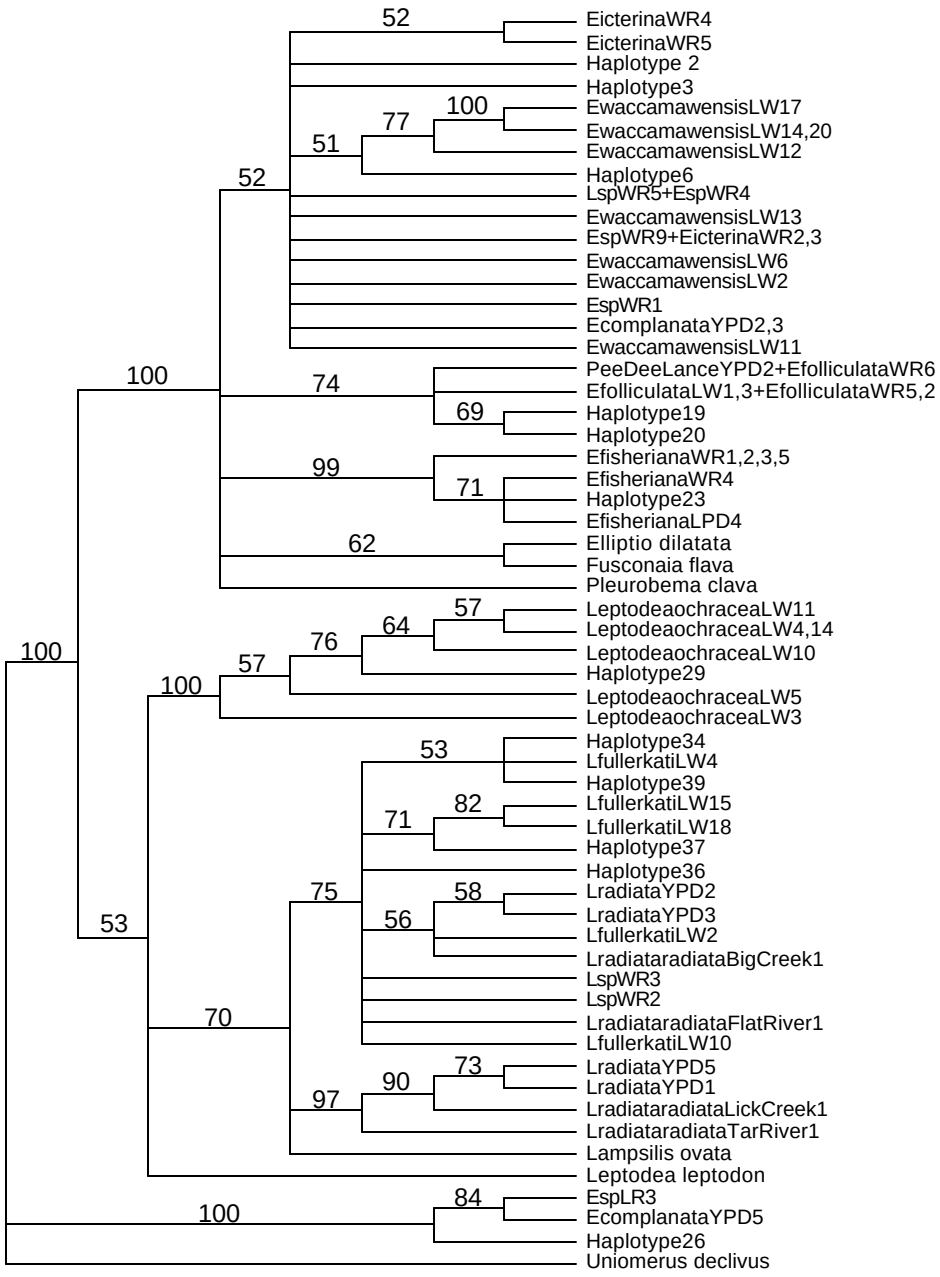


Figure 4b. Bootstrap consensus of 16s sequence data. See Fig. 4a legend for details.

classified morphologically as *E. complanata*, and 2 as unidentified *Elliptio*. The sequences of these individuals (clade G in Fig 4a) were very distinct from 2 other *E. complanata* from the Pee Dee (see below), which grouped with other *Elliptio* within the Pleuroblemini. The 7 distinctive sequences did not group with *Elliptio* nor did they fall within the Pleuroblemini, but instead formed a clade that was basal. This monophyletic, cryptic species may be a *Uniomerus*, which can be confused with *Elliptio* in surveys (we thank Karen Lynch of NCDOT for alerting us to this possibility). We collected *Uniomerus carolinianus* from the Lumber and Little Pee Dee Rivers, and are presently sequencing these animals to evaluate whether they may group with the misidentified animals.

The other Waccamaw endemic, *Lampsilis fullerkati*, fell within a clade (**E**) that also included *Lampsilis radiata*, and *Lampsilis radiata radiata* from outside of the lake, so again, the endemic status of this species was not supported. The clade containing this endemic, however, was very distinct from the clade containing a specimen (collected by J. Alderman in 2001) of *Lampsilis radiata radiata* from Lick Creek in the Pee Dee, and an specimen of *Lampsilis* sp. of ongoing systematic interest (J. Alderman, pers. comm.) from the upper Tar River. Of particular interest in this regard is our finding that 2 genetically distinctive forms from our collection, morphologically called *L. radiata*, coexist in the Pee Dee River—one form (individuals YPD1 and YPD5) that groups with *Lampsilis* sp. from the upper Tar, and the other form (individuals YPD2, YPD3, and YPD4) that groups with *L. radiata radiata* and *L. fullerkati*.

Table 8. 16s haplotypes shared by multiple individuals

Haplotype	Individuals		
2	EcongaraeaWR1,2,3,4,5	EwaccamawensisLW1,3,4,5,7,8	EwaccamawensisWR2,3,6,9
	EspWR3,5,6,7	UnknownLW1	
3	EicterinaWR1	EwaccamawensisWR1,4,5,7	EspLR5
6	EwaccamawensisLW9	EwaccamawensisWR8,10	LspLR4,6
19	EfolliculataWR8,9	EfolliculataLW2	EproductaWR1
20	EfolliculataWR1,3,4,7,10	EproductaWR2,3,4,5	PeeDeeLanceYPD1,3
	EspYPD1,2,3,4		
23	EfisherianaLPD1,2,3,5	EfisherianaWR6,7,8,9,10	
26	*EcomplanataLR1,2,3,4,5	*EcomplanataYPD1,4	*EspLR1,2,4
29	LepochraceaLW1,2,7,8,9,12,13,15		
34	LfullerkatiLW5,6,7,8,16,17		
36	LfullerkatiLW1,3,11,12,14,19,20	LradiataLR4	
37	LfullerkatiLW13	LradiataradiataWR1	LspLR1
39	LepochraceaLW6	LfullerkatiLW9	LradiataradiataWR2
	LspLR7		

In general, there was poor resolution on the 16s tree for closely related taxa. Analysis of the CO I region (Figs. 5) did show more phylogenetic signal. Two subclades were resolved in both *E. fisheriana* and in the lances, but the taxonomic or biogeographic significance of these is unclear. More to the point of this project, all *E. waccamawensis* from Lake Waccamaw were confined to a single subclade (**F**) nested within a larger clade (**E + F**). Subclade F, however, contained *E. congaraea* and *E. waccamawensis* from both the Lake and the Waccamaw River, with no phylogenetic structure with respect to species or

geographic source. Hence even with the apparently greater resolution, we still found no evidence that *E. waccamawensis* from Lake Waccamaw is a phylogenetic species.

Figure 5a. COI Phylogeny

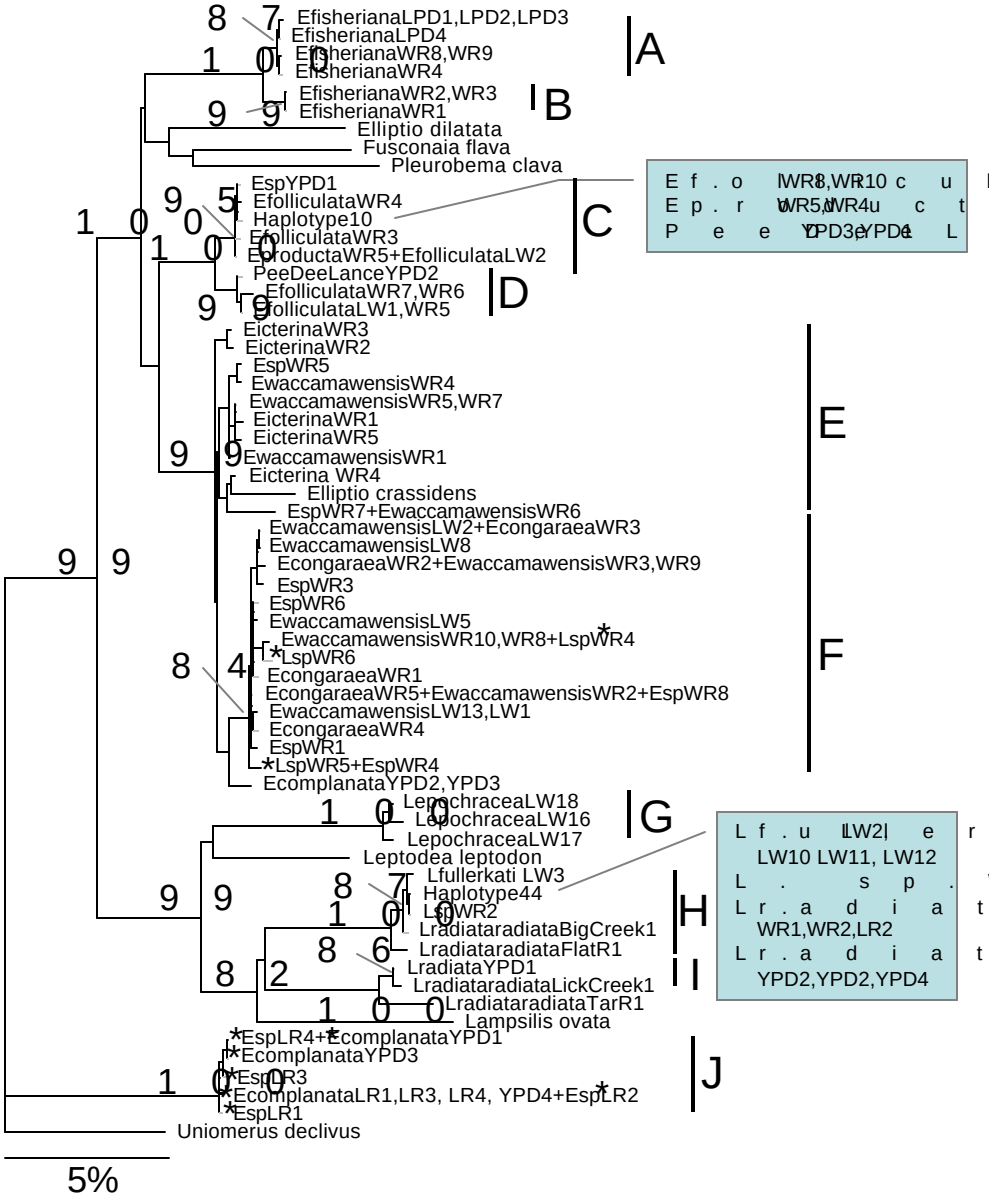


Figure 5a. Neighbor-joining phylogram of COI sequence data. Numbers on branches are bootstrap support (1000 NJ replications). Values less than 70 are not shown, and some values for subclades are not shown; see Fig. 5b for all values. Callout boxes show individuals that share identical, numbered haplotypes. Taxa are labeled with morphological species (asterisks mark likely misidentifications), followed by location abbreviation (LW=Lake Waccamaw, WR= Waccamaw River, YPD= Yadkin-Pee Dee River, LPD=Little Pee Dee River), followed by individual ID #. Commas separate individuals from the same location that share haplotypes. Vertical bars with bolded letters designate well supported clades, or phylogroups discussed in the text.

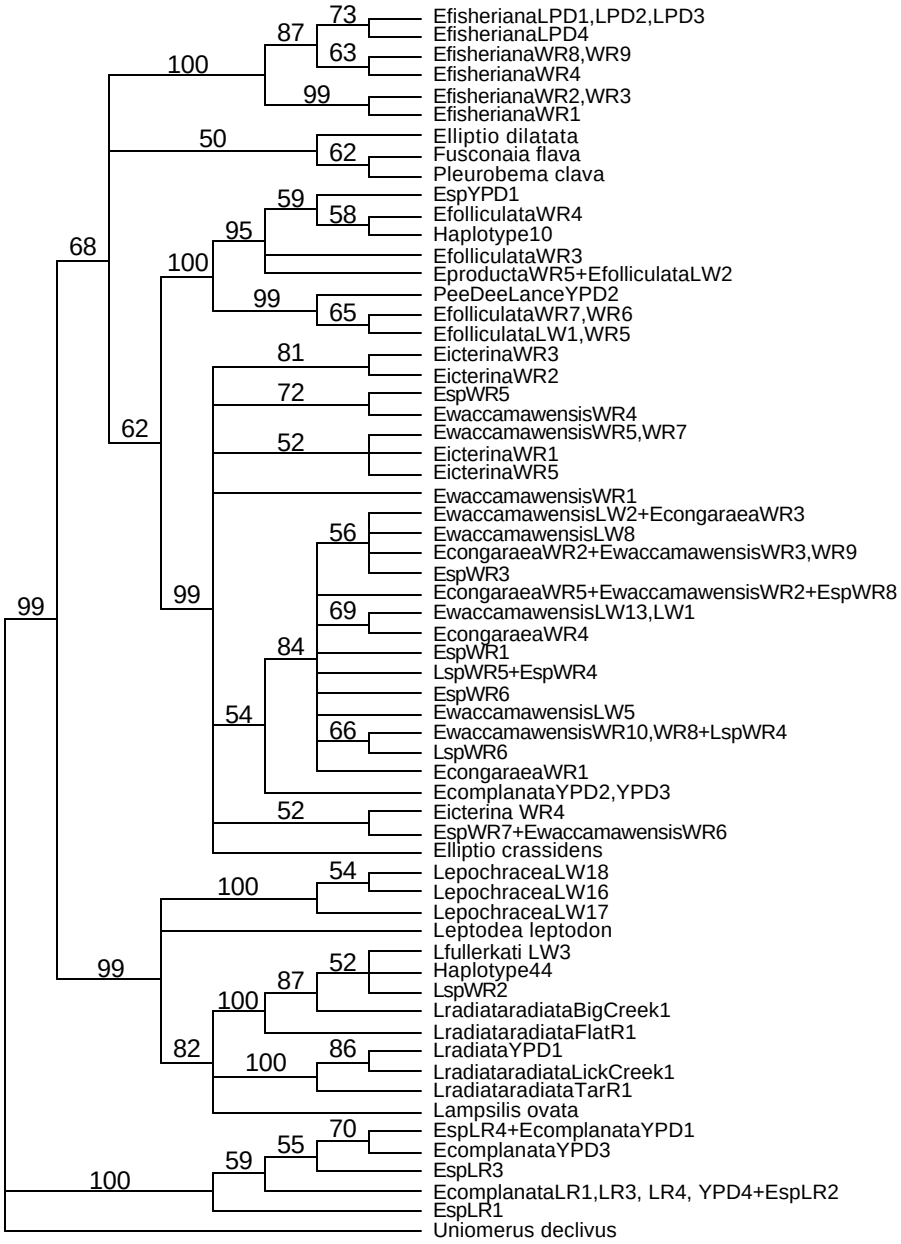


Figure 5b. Bootstrap consensus of COI sequence data. See Fig. 5a legend for details.

Other major groupings within the COI tree were mostly concordant with those found in the 16s tree. In particular, the CO I tree also resolved the two major lineages of *Lampsilis* sp. and *Lampsilis radiata* and showed that the animals from the upper Tar, from Lick Creek, and from our collection in the Yadkin-Pee Dee were distinct. And as in the 16s tree, there was no evidence that *Lampsilis fullerkati* forms a monophyletic endemic lineage; again it shared identical haplotypes with and/or was intermingled with sequences from outside of

the Lake. At present, these results suggest that the two Lake Waccamaw endemics are not phylogenetic species, but they do indicate interesting affinities with more widespread species. For instance, *E. waccamawensis* appears to be mostly closely related to *E. congaraea*, and this warrants closer scrutiny in a biogeographic study. And the affinities of *L. fullerkati* seem to lie with one major lineage within *Lampsilis radiata*. Both of these findings must be evaluated using the type specimens that we are currently sequencing.

As a final application of our molecular markers to describing and distinguishing freshwater mussel species in North Carolina, we focused on the two cases of cryptic taxa revealed in the phylogenetic analyses. First, we calculated the degree of genetic distance (calculated using the Tamura-Nei model) between the cryptic taxon and the species that it was identified as. The first case is the unknown animals that were identified in our collections as *Elliptio complanata.* Mean genetic distances between these individuals and the other *Elliptio complanata* that group within the genus *Elliptio* was about 14% for both the 16s and CO I regions. This large distance, like the phylogeny, suggests a distant relationship between this cryptic taxon and *Elliptio complanata*. The second case concerns a cryptic taxon of *Lampsilis*. We calculated that the mean % difference between *Lampsilis radiata radiata*/*Lampsilis fullerkati* and the other lineage of *Lampsilis* sp. containing specimens of this cryptic taxon from the upper Tar and Pee Dee, and this distance was 5.6 and 8.7 % for 16s and CO I regions, respectively. Again, this indicates a substantial genetic separation between these taxa.

Sequence divergence in both cases was large enough to allow us to design diagnostic assays, based on restriction enzyme digestion with multiple enzymes, which can be used to differentiate the taxa (Table 9, 10). We have selected a minimum of 2 enzymes per gene per species pair that each show diagnostic cut sites. These assays will provide biologists sampling in, for example, the Pee Dee system, an additional means to distinguish between these morphologically very similar animals.

Table 9a. Diagnostic Restriction Enzymes for distinguishing cryptic taxa based on 16s. Fragment sizes are in base pairs.

Gene region	Enzyme	Taxon	Number of sites	Fragments
16s rDNA	Tsp 5091	*E. complanata*	1	84, 388
		E. complanata-like amblemid	3	84, 56, 160, 172
	Mse I	*E. complanata*	2	83, 316, 73
		E. complanata-like amblemid	5 or 6	31, 52, 51, 39, 51, 175, 73
	EcoR V	*Lampsilis radiata/fullerkati*	2	118, 315, 66
		Lampsilis sp. "Tar River"	1	433, 66
	Rsa I	*Lampsilis radiata/fullerkati*	2	124, 264, 111
		Lampsilis sp. "Tar River"	3	124, 93, 171, 111

Table 9b. Diagnostic Restriction Enzymes for distinguishing cryptic taxa based on COI. Fragment sizes are in base pairs.

Gene region	Enzyme	Taxon	Number of sites	Fragments
CO I	Dra II	*E. complanata*	1	200, 422
		E. complanata-like amblemid	0	622
	EcoR I	*E. complanata*	0	622
		E. complanata-like amblemid	1	476, 146
	Bsr I	*E. complanata*	0	622
		E. complanata-like amblemid	1	307, 315
	Hinf I	*Lampsilis radiata/fullerkati*	1 or 2	49, 9, 577
		Lampsilis sp. "Tar River"	2	49, 441, 145
	Rsa I	*Lampsilis radiata/fullerkati*	0	635
		Lampsilis sp. "Tar River"	1	267, 368

IV. Findings and conclusions

Our results show that hemolymph sampling provides a non-lethal and efficient method for sampling freshwater mussels. Hemolymph removal produced no mortality and minimal impacts on growth when assessed over an 8-week period. The technique is easily applied in the field and can be accomplished with minimal training. Routine collection of hemolymph by field biologists could provide a tissue sample archive that would represent an invaluable resource that, analyzed with rapid genetic identification methods like those we employed, and supplemented with an ever-increasing DNA sequence database, would provide confirmation of field identifications of freshwater mussels based on shell characters or soft tissue traits.

Our data also suggest that the mtDNA markers 16s and COI provide sufficient levels of interspecific differentiation to allow the development of PCR-

RFLP assays to distinguish among several taxa. PCR-RFLP assays have the advantage over sequence based genetic analysis in that the diagnostics are less expensive and require less instrumentation. Thus, PCR-RFLP assays are suitable for use in most labs, and their broad scale implementation would allow multiple labs to participate in mapping distributions of species statewide.

This project represents our first experience with freshwater mussel taxonomy and phylogeny, and also served as the basis for the training of a graduate student with no prior background in biology or taxonomy of freshwater mussels. Without the help of Dr. Heise and Mr. Nichols in the field, we could not have hoped to identify the animals we collected. The data suggest that nearly all of their identifications were "correct," and there are only a limited number of specimens that upon genetic typing, we determined must represent misidentifications. So we emphasize that the methods described in this report are in no way intended to replace morphological taxonomy, nor do they substitute for experienced of biologists trained in freshwater mussel taxonomy. Our methods are simply an alternative tool, an independent source of information that has the additional advantage of being available when morphological classification is ambiguous.

We were unable to analyze type specimens for several of the species that we collected from the regional survey of the Lumber and Yadkin-Pee Dee River Basins, prior to submitting this report. Names for the taxa on our trees is therefore provisional, and must await the data from type specimens (which we are now helping to gather) for verification. Both *Elliptio* and *Lampsilis*, moreover, will undergo considerable revision in the coming years. It is obvious to us that that this work must be done by systematicists, and that it is well outside of our expertise and beyond the scope of this project. Nevertheless, the names we used were derived from careful examination of shell characters and comparison to current taxonomic keys by field biologists. Experienced biologists who routinely survey natural populations in the state, and who face the challenge of monitoring populations of these taxonomically very challenging animals made the identifications. Then we added the DNA data to attach to these provisional taxa. The last step is to include data from type specimens as these data become available. Use of provisional biological nomenclature in this manner to suggest hypotheses about a fauna that has previously not been analyzed using DNA data seems reasonable to us. For example, for only a fraction of the species in Campbell et al. (2005) were DNA sequences available from type specimens.

So the present report is certainly a work in progress. Nevertheless, we have enough information at hand to confidently address one of our three primary research questions, and that is the issue of the phylogenetic species status of the Lake Waccamaw endemics. Neither of these described species forms a monophyletic group exclusively containing Lake Waccamaw specimens. In the case of *E. waccamawensis*, our current hypothesis is that this animal is an ecomorph of an animal we collected from the Waccamaw River and that our

colleagues identified as *E. congaraea;* in a similar manner, *L. fullerkati* appears to be genetically indistinguishable from a lineage of *L. radiata* (subspecies *radiata?*) from other locations. Addition of new specimens, outgroup taxa and type specimens included, will not create monophyly for the Lake Waccamaw animals, it will affect how these and related taxa are named and more clearly define their geographic distributions. Examination of gene flow based on hypervariable DNAs, such as microsatellites, could provide an alternative evaluation, particularly to address genetic and reproductive isolation between the Lake and adjacent populations in the Waccamaw River. It is also possible that nuclear DNA sequences, or even sequences from other portions of the mtDNA, might reveal that the Lake endemics are unique, but our results suggest that they are at best, very closely related to and nested within a more widespread species.

So at present, our work in Lake Waccamaw, the adjacent Waccamaw River, and in tributaries in the Lumber and Yadkin Pee Dee River Basins, suggests that despite the traditional interpretation of morphological features, *Elliptio waccamawensis* and *Lampsilis fullerkati* and are not strictly lake-endemics. Whether or not the two taxa to which these animals belong are restricted, to the Lumber River Basin (of which Lake Wacamaw is a part), to the larger Yadkin-Pee Dee River drainage, or are even more broadly distributed will require a more extensive survey. While our study did not support the endemic status of the Lake forms, we did find evidence for other cryptic taxa that co-occur with related species.

The approach used in this project has both strengths and limitations. Hemolymph extraction is an easy way to obtain tissue from these animals, it is easy to train field assistants how to use the technique, and it takes as little or less time to accomplish than mantle clipping. It also produced no mortality in our hands, although the diminution in growth we observed should be followed up with longer-term studies. We cannot comment on whether mantle sampling presents a greater risk to the animals; this would also be worth evaluating in future work, since mantle is in our hands a slightly more efficient source for DNA extraction (that is, it produces fewer negative results in PCR).

We agree with reviewers who voiced reservations about the application of the hemolymph sampling to studies of the systematics of these animals, and we agree with reviewer comments that emphasized the problems with sampling an animal for which no voucher of the shell or soft tissues is deposited. In our case, our inclination was to be as conservative as possible and to harvest as few specimens as we could. The drawback was that in the cases where a misidentification or unknown taxon was detected by DNA analysis, we sometimes had no voucher specimen and only the photographs, which we learned (as others already know) to not be adequate for reexamining the specimen. For example, we have no vouchers of the animals from Morrow Mountain State Park that appeared to be *Elliptio complanata*, yet are clearly not members of this genus. Our conclusion is that non lethal tissue snipping and

hemolymph sampling can most effectively contribute genetic data to be used to compare to a large database of mtDNA sequences that is referenced to deposited specimens. Such is the spirit of the "DNA barcoding" approach to conservation genetics (e.g. Dasmahapatra and Mallet 2006; Hebert et al. 2004; Hogg and Hebert 2004), which we believe could be a very useful approach in the case of freshwater mussels, and which we believe could be accomplished in part using the methods we investigated here. We provide a few recommendations on how this might work, below.

G. Recommendations for Implementation and Technology Transfer

Our work provides an outline for standard procedures that could be used in genetic identification of freshwater mussel species in North Carolina. We have developed field methods for hemolymph sampling that do not result in mortality of mussels and that provide a tissue sample suitable for DNA extraction. Such methods could be easily incorporated into field survey protocols. In partnership with individuals working at universities, other state agencies, and private consulting firms, these methods could be used to help produce a genetic database that would clarify our understanding of the distributions and relationships of NC freshwater mussel resources. Our recommendation for how routine, non-lethal genetic identification might fit into the scheme of freshwater mussel survey work and research in North Carolina is below:

(1) The database of DNA sequences would be public access, and sequences in that database would be cross-referenced to voucher specimens deposited at the North Carolina Museum of Natural Sciences.

(2) Biologists involved in monitoring natural populations would routinely collect hemolymph from field-collected animals. This tissue can be stored on ice in the field and transferred to laboratories at the agencies or partner institutions that are conducting the molecular analysis.

(3) PCR-RFLP assays would be conducted on the hemolymph samples, producing a rapid initial identification that would be compared to the morphological identification. Inconsistencies would be flagged for DNA sequencing work. In addition, the molecular lab would routinely sequence a subsample of the submitted samples, or any that had been flagged in the field for examination (from say priority sampling sites).

(4) All DNA sequences would be submitted to a different database for cross comparison to the master taxonomic database. Particular issues would arise in the case of query sequences that are not in the master database. In this case, the record of the specimen and its GPS coordinates would be retrieved, and a decision would be made whether to revisit the site and to collect the whole animal for submission to the museum collection, taxonomic work, and DNA sequencing. As an alternative in the case of endangered populations or when

population viability is unknown, state biologists could consider the option of collecting hemolymph, tagging the animal, and repeating DNA analysis.

This outline can be much improved, of course. Linking this to existing resources, such as the databases of the National Heritage Program, is an example of how it might be implemented. Nevertheless, we believe that hemolymph collected as part of routine survey work, subjected to PCR-RFLP analysis and DNA sequence analysis of subsamples, would improve the database for understanding the systematics and geographic distribution of freshwater mussels in the state. Using modifications of the methods we have developed, data can be collected quickly and with minimal risk to the animals.

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Appendix 1. Results: effects of hemolymph extraction on growth and survivorship of Lake Waccamaw mussels

Y = yes, N = no, NR = not recovered

Date	Genus	Enclosure	Tag color	ID#	Length (mm)	Extracted	Alive	Growth (? mm)
4/7/04	Elliptio	1	Aqua	40	57.85	Y	Y	
6/2/04	Elliptio	1	Aqua	40	58.35	Y	Y	0.50
4/7/04	Elliptio	1	Aqua	41	50.06	Y	Y	
6/2/04	Elliptio	1	Aqua	41	50.48	Y	Y	0.42
4/7/04	Elliptio	1	Aqua	42	52.70	Y	Y	
6/2/04	Elliptio	1	Aqua	42	52.73	Y	Y	0.03
4/7/04	Elliptio	1	Aqua	43	53.39	Y	Y	
6/2/04	Elliptio	1	Aqua	43	53.52	Y	Y	0.13
4/7/04	Elliptio	1	Aqua	44	54.08	Y	Y	
6/2/04	Elliptio	1	Aqua	44	54.50	Y	Y	0.42
4/7/04	Elliptio	1	Aqua	45	47.02	Y	Y	
6/2/04	Elliptio	1	Aqua	45	47.51	Y	Y	0.49
4/7/04	Elliptio	1	Aqua	46	49.94	Y	Y	
6/2/04	Elliptio	1	Aqua	46	50.71	Y	Y	0.77
4/7/04	Elliptio	1	Aqua	47	44.55	Y	Y	
6/2/04	Elliptio	1	Aqua	47	44.55	Y	Y	0.00
4/7/04	Elliptio	1	Aqua	48	48.42	Y	Y	
6/2/04	Elliptio	2	Aqua	48	48.91	Y	Y	0.49
4/7/04	Elliptio	1	Aqua	50	58.55	Y	Y	
6/2/04	Elliptio	1	Aqua	50	59.07	Y	Y	0.52
4/7/04	Elliptio	2	Aqua	51	56.77	Y	Y	
6/2/04	Elliptio	2	Aqua	51	57.53	Y	Y	0.76
4/7/04	Elliptio	2	Aqua	52	57.22	Y	Y	
6/2/04	Elliptio	2	Aqua	52	57.44	Y	Y	0.22
4/7/04	Elliptio	2	Aqua	53	40.69	Y	Y	
6/2/04	Elliptio	2	Aqua	53	41.34	Y	Y	0.65
4/7/04	Elliptio	2	Aqua	54	51.60	Y	Y	
6/2/04	Elliptio	2	Aqua	54	52.37	Y	Y	0.77
4/7/04	Elliptio	2	Aqua	55	53.93	Y	Y	
6/2/04	Elliptio	2	Aqua	55	54.51	Y	Y	0.58
4/7/04	Elliptio	2	Aqua	56	47.02	Y	Y	
6/2/04	Elliptio	2	Aqua	56	47.67	Y	Y	0.65
4/7/04	Elliptio	2	Aqua	57	52.35	Y	Y	
6/2/04	Elliptio	2	Aqua	57	52.82	Y	Y	0.47
4/7/04	Elliptio	2	Aqua	58	45.18	Y	Y	
6/2/04	Elliptio	2	Aqua	58	45.76	Y	Y	0.58
Date	Genus	Enclosure	Tag color	ID#	Length (mm)	Extracted	Alive	Growth (? mm)

4/7/04	Elliptio	2	Aqua	59	44.61	Y	Y	
6/2/04	Elliptio	2	Aqua	59	45.98	Y	Y	1.37
4/7/04	Elliptio	2	Aqua	60	45.68	Y	Y	
6/2/04	Elliptio	2	Aqua	60	47.17	Y	Y	1.49
4/7/04	Elliptio	1	Aqua	61	45.48	N	Y	
6/2/04	Elliptio	1	Aqua	61	47.49	N	Y	2.01
4/7/04	Elliptio	1	Aqua	62	43.47	N	Y	
6/2/04	Elliptio	1	Aqua	62	44.37	N	Y	0.90
4/7/04	Elliptio	1	Aqua	63	47.58	N	Y	
6/2/04	Elliptio	1	Aqua	63	47.75	N	Y	0.17
4/7/04	Elliptio	1	Aqua	64	51.10	N	Y	
6/2/04	Elliptio	1	Aqua	64	51.53	N	Y	0.43
4/7/04	Elliptio	1	Aqua	65	38.59	N	Y	
6/2/04	Elliptio	1	Aqua	65	39.20	N	Y	0.61
4/7/04	Elliptio	1	Aqua	66	42.44	N	Y	
6/2/04	Elliptio	1	Aqua	66	42.89	N	Y	0.45
4/7/04	Elliptio	1	Aqua	67	42.47	N	Y	
6/2/04	Elliptio	1	Aqua	67	42.88	N	Y	0.41
4/7/04	Elliptio	1	Aqua	68	47.47	N	Y	
6/2/04	Elliptio	1	Aqua	68	48.53	N	Y	1.06
4/7/04	Elliptio	1	Aqua	69	45.85	N	Y	
6/2/04	Elliptio	1	Aqua	69	46.56	N	Y	0.71
4/7/04	Elliptio	1	Aqua	70	44.02	N	Y	
6/2/04	Elliptio	1	Aqua	70	44.25	N	Y	0.23
4/7/04	Elliptio	2	Aqua	71	52.69	N	Y	
6/2/04	Elliptio	2	Aqua	71	53.10	N	Y	0.41
4/7/04	Elliptio	2	Aqua	72	50.03	N	Y	
6/2/04	Elliptio	2	Aqua	72	49.88	N	Y	-0.15
4/7/04	Elliptio	2	Aqua	73	52.51	N	Y	
6/2/04	Elliptio	2	Aqua	73	52.53	N	Y	0.02
4/7/04	Elliptio	2	Aqua	74	54.93	N	Y	
6/2/04	Elliptio	2	Aqua	74	55.55	N	Y	0.62
4/7/04	Elliptio	2	Aqua	75	42.02	N	Y	
6/2/04	Elliptio	2	Aqua	75	42.29	N	Y	0.27
4/7/04	Elliptio	2	Aqua	76	49.90	N	Y	
6/2/04	Elliptio	2	Aqua	76	50.32	N	Y	0.42
4/7/04	Elliptio	2	Aqua	77	49.32	N	Y	
6/2/04	Elliptio	2	Aqua	77	0.00	N	NR	
4/7/04	Elliptio	2	Aqua	78	54.36	N	Y	
6/2/04	Elliptio	2	Aqua	78	55.62	N	Y	1.26
Date	Genus	Enclosure	Tag color	ID#	Length (mm)	Extracted	Alive	Growth (? mm)
4/7/04	Elliptio	2	Aqua	79	51.02	N	Y	
6/2/04	Elliptio	2	Aqua	79	52.29	N	Y	1.27

4/7/04	Elliptio	2	Aqua	80	42.30	N	Y	
6/2/04	Elliptio	2	Aqua	80	43.46	N	Y	1.16
4/7/04	Lampsilis	1	Orange	40	42.47	Y	Y	
6/2/04	Lampsilis	1	Orange	40	42.57	Y	Y	0.10
4/7/04	Lampsilis	1	Orange	41	54.85	Y	Y	
6/2/04	Lampsilis	1	Orange	41	55.79	Y	Y	0.94
4/7/04	Lampsilis	1	Orange	42	43.03	Y	Y	
6/2/04	Lampsilis	1	Orange	42	?	Y	Y	
4/7/04	Lampsilis	1	Orange	43	49.82	Y	Y	
6/2/04	Lampsilis	1	Orange	43	50.65	Y	Y	0.83
4/7/04	Lampsilis	1	Orange	44	50.24	Y	Y	
6/2/04	Lampsilis	1	Orange	44	50.93	Y	Y	0.69
4/7/04	Lampsilis	1	Orange	45	43.63	Y	Y	
6/2/04	Lampsilis	1	Orange	45	43.84	Y	Y	0.21
4/7/04	Lampsilis	1	Orange	46	45.94	Y	Y	
6/2/04	Lampsilis	1	Orange	46	46.72	Y	Y	0.78
4/7/04	Lampsilis	1	Orange	47	52.28	Y	Y	
6/2/04	Lampsilis	1	Orange	47	52.45	Y	Y	0.17
4/7/04	Lampsilis	2	Orange	48	48.27	Y	Y	
6/2/04	Lampsilis	2	Orange	48	48.78	Y	Y	0.51
4/7/04	Lampsilis	2	Orange	49	42.33	Y	Y	
6/2/04	Lampsilis	2	Orange	49	42.56	Y	Y	0.23
4/7/04	Lampsilis	2	Orange	50	46.24	Y	Y	
6/2/04	Lampsilis	2	Orange	50	46.43	Y	Y	0.19
4/7/04	Lampsilis	2	Orange	51	48.74	Y	Y	
6/2/04	Lampsilis	2	Orange	51	49.46	Y	Y	0.72
4/7/04	Lampsilis	2	Orange	52	53.67	Y	Y	
6/2/04	Lampsilis	2	Orange	52	53.80	Y	Y	0.13
4/7/04	Lampsilis	2	Orange	53	46.90	Y	Y	
6/2/04	Lampsilis	2	Orange	53	47.40	Y	Y	0.50
4/7/04	Lampsilis	2	Orange	54	46.73	Y	Y	
6/2/04	Lampsilis	2	Orange	54	46.84	Y	Y	0.11
4/7/04	Lampsilis	2	Orange	55	52.07	Y	Y	
6/2/04	Lampsilis	2	Orange	55	52.23	Y	Y	0.16
4/7/04	Lampsilis	1	Orange	56	48.94	N	Y	
6/2/04	Lampsilis	1	Orange	56	49.06	N	Y	0.12
4/7/04	Lampsilis	1	Orange	57	46.78	N	Y	
6/2/04	Lampsilis	1	Orange	57	47.79	N	Y	1.01
Date	Genus	Enclosure	Tag color	ID#	Length (mm)	Extracted	Alive	Growth (? mm)
4/7/04	Lampsilis	1	Orange	58	51.13	N	Y	
6/2/04	Lampsilis	1	Orange	58	51.97	N	Y	0.84
4/7/04	Lampsilis	1	Orange	59	45.87	N	Y	
6/2/04	Lampsilis	1	Orange	59	46.71	N	Y	0.84

4/7/04	Lampsilis	1	Orange	60	44.80	N	Y	
6/2/04	Lampsilis	2	Orange	60	45.01	N	Y	0.21
4/7/04	Lampsilis	1	Orange	61	56.43	N	Y	
6/2/04	Lampsilis	1	Orange	61	56.53	N	N	
4/7/04	Lampsilis	1	Orange	62	45.91	N	Y	
6/2/04	Lampsilis	1	Orange	62	46.35	N	Y	0.44
4/7/04	Lampsilis	1	Orange	63	48.55	N	Y	
6/2/04	Lampsilis	1	Orange	63	48.95	N	Y	0.40
4/7/04	Lampsilis	2	Orange	64	49.75	N	Y	
6/2/04	Lampsilis	2	Orange	64	49.97	N	Y	0.22
4/7/04	Lampsilis	2	Orange	65	43.56	N	Y	
6/2/04	Lampsilis	2	Orange	65	43.63	N	Y	0.07
4/7/04	Lampsilis	2	Orange	66	47.66	N	Y	
6/2/04	Lampsilis	2	Orange	66	48.04	N	Y	0.38
4/7/04	Lampsilis	2	Orange	67	48.53	N	Y	
6/2/04	Lampsilis	2	Orange	67	48.78	N	Y	0.25
4/7/04	Lampsilis	2	Orange	68	47.22	N	Y	
6/2/04	Lampsilis	2	Orange	68	47.93	N	Y	0.71
4/7/04	Lampsilis	2	Orange	69	49.04	N	Y	
6/2/04	Lampsilis	2	Orange	69	49.30	N	Y	0.26
4/7/04	Lampsilis	2	Orange	70	48.61	N	Y	
6/2/04	Lampsilis	2	Orange	70	49.15	N	Y	0.54
4/7/04	Lampsilis	2	Orange	71	42.74	N	Y	
6/2/04	Lampsilis	2	Orange	71	43.21	N	Y	0.47
4/7/04	Leptodea	1	White	40	44.28	Y	Y	
6/2/04	Leptodea	1	White	40	47.30	Y	Y	3.02
4/7/04	Leptodea	1	White	41	45.15	Y	Y	
6/2/04	Leptodea	1	White	41	46.54	Y	Y	1.39
4/7/04	Leptodea	1	White	42	45.27	Y	Y	
6/2/04	Leptodea	1	White	42	48.52	Y	Y	3.25
4/7/04	Leptodea	1	White	43	45.95	Y	Y	
6/2/04	Leptodea	1	White	43	49.15	Y	Y	3.20
4/7/04	Leptodea	1	White	44	35.98	Y	Y	
6/2/04	Leptodea	1	White	44	39.38	Y	Y	3.40
4/7/04	Leptodea	1	White	45	33.27	Y	Y	
6/2/04	Leptodea	1	White	45	0.00	Y	NR	
Date	Genus	Enclosure	Tag color	ID#	Length (mm)	Extracted	Alive	Growth (? mm)
4/7/04	Leptodea	1	White	46	47.80	Y	Y	
6/2/04	Leptodea	1	White	46	50.75	Y	Y	2.95
4/7/04	Leptodea	1	White	47	44.78	Y	Y	
6/2/04	Leptodea	1	White	47	47.46	Y	Y	2.68
4/7/04	Leptodea	1	White	48	46.67	Y	Y	
6/2/04	Leptodea	1	White	48	50.02	Y	Y	3.35

4/7/04	Leptodea	1	White	49	46.45	Y	Y	
6/2/04	Leptodea	1	White	49	49.94	Y	Y	3.49
4/7/04	Leptodea	2	White	50	45.52	Y	Y	
6/2/04	Leptodea	2	White	50	0.00	Y	NR	
4/7/04	Leptodea	2	White	51	45.42	Y	Y	
6/2/04	Leptodea	2	White	51	48.95	Y	Y	3.53
4/7/04	Leptodea	2	White	52	43.65	Y	Y	
6/2/04	Leptodea	2	White	52	47.41	Y	Y	3.76
4/7/04	Leptodea	2	White	53	51.59	Y	Y	
6/2/04	Leptodea	2	White	53	54.89	Y	Y	3.30
4/7/04	Leptodea	2	White	54	50.66	Y	Y	
6/2/04	Leptodea	2	White	54	52.68	Y	Y	2.02
4/7/04	Leptodea	2	White	55	44.20	Y	Y	
6/2/04	Leptodea	2	White	55	48.52	Y	Y	4.32
4/7/04	Leptodea	2	White	56	45.56	Y	Y	
6/2/04	Leptodea	2	White	56	48.52	Y	Y	2.96
4/7/04	Leptodea	2	White	57	44.65	Y	Y	
6/2/04	Leptodea	2	White	57	47.54	Y	Y	2.89
4/7/04	Leptodea	2	White	58	44.32	Y	Y	
6/2/04	Leptodea	2	White	58	46.31	Y	Y	1.99
4/7/04	Leptodea	2	White	59	40.66	Y	Y	
6/2/04	Leptodea	2	White	59	0.00	Y	NR	
4/7/04	Leptodea	1	White	60	43.90	N	Y	
6/2/04	Leptodea	1	White	60	46.75	N	Y	2.85
4/7/04	Leptodea	1	White	61	41.83	N	Y	
6/2/04	Leptodea	1	White	61	46.01	N	Y	4.18
4/7/04	Leptodea	1	White	62	40.11	N	Y	
6/2/04	Leptodea	1	White	62	43.35	N	Y	3.24
4/7/04	Leptodea	1	White	63	37.38	N	Y	
6/2/04	Leptodea	1	White	63	0.00	N	NR	
4/7/04	Leptodea	1	White	64	43.13	N	Y	
6/2/04	Leptodea	1	White	64	46.12	N	Y	2.99
4/7/04	Leptodea	1	White	65	40.09	N	Y	
6/2/04	Leptodea	1	White	65	43.75	N	Y	3.66
Date	Genus	Enclosure	Tag color	ID#	Length (mm)	Extracted	Alive	Growth (? mm)
4/7/04	Leptodea	1	White	66	44.84	N	Y	
6/2/04	Leptodea	1	White	66	48.14	N	Y	3.30
4/7/04	Leptodea	1	White	67	38.13	N	Y	
6/2/04	Leptodea	1	White	67	41.01	N	Y	2.88
4/7/04	Leptodea	1	White	68	44.02	N	Y	
6/2/04	Leptodea	1	White	68	46.89	N	Y	2.87
4/7/04	Leptodea	1	White	69	41.89	N	Y	
6/2/04	Leptodea	1	White	69	45.03	N	Y	3.14

4/7/04	Leptodea	2	White	70	35.13	N	Y	
6/2/04	Leptodea	2	White	70	0.00	N	NR	
4/7/04	Leptodea	2	White	71	34.67	N	Y	
6/2/04	Leptodea	2	White	71	0.00	N	NR	
4/7/04	Leptodea	2	White	72	44.25	N	Y	
6/2/04	Leptodea	2	White	72	45.96	N	Y	1.71
4/7/04	Leptodea	2	White	73	46.57	N	Y	
6/2/04	Leptodea	2	White	73	50.91	N	Y	4.34
4/7/04	Leptodea	2	White	74	39.00	N	Y	
6/2/04	Leptodea	2	White	74	0.00	N	NR	
4/7/04	Leptodea	2	White	75	42.22	N	Y	
6/2/04	Leptodea	2	White	75	45.35	N	Y	3.13
4/7/04	Leptodea	2	White	76	48.55	N	Y	
6/2/04	Leptodea	2	White	76	51.94	N	Y	3.39
4/7/04	Leptodea	2	White	78	37.99	N	Y	
6/2/04	Leptodea	2	White	78	41.27	N	Y	3.28
4/7/04	Leptodea	2	White	79	41.19	N	Y	
6/2/04	Leptodea	2	White	79	43.70	N	Y	2.51
4/7/04	Leptodea	2	White	80	40.62	N	Y	
6/2/04	Leptodea	2	White	80	44.33	N	Y	3.71

Appendix 2. List of specimens and tissue samples

Date	Location	Latitude Longitude	County	River	Drainage	Morphological species	# Hemolymphs	# Vouchers
09/18/01	Below Lake Wacc Dam	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis radiata radiata*	2	2
09/18/01	Lick Creek SR1246	34º57'N 80º14'W?	Anson	Pee Dee	Yadkin-Pee Dee	*Lampsilis* sp.	1	1
09/18/01	Tar River SR1138	36º12'N 78º33'W?	Granville	Tar	Tar-Pamlico	*Lampsilis* sp.	1	1
09/18/01	Flat River SR1471	36º14'N 78º54'W?	Durham	Flat	Neuse	*Lampsilis radiata radiata*	1	1
09/25/01	Big Creek, L. Waccamaw	34º17'N 78º28'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis radiata radiata*	1	1
4/7/04	Lake Waccamaw	34º16'N 78º28'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Elliptio waccamawensis*	20	42
4/7/04	Lake Waccamaw	34º16'N 78º28'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis fullerkati*	16	30
4/7/04	Lake Waccamaw	34º16'N 78º28'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Leptodea ochracea*	20	40
8/10/04	North Shore L. Waccamaw	34º18'N 78º30'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Leptodea ochracea*	20	0
8/10/04	Above Dam L. Waccamaw	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Leptodea ochracea*	10	2

Date	Location	Latitude Longitude	County	River	Drainage	Morphological species	# Hemolymphs	# Vouchers
8/10/04	North Shore L. Waccamaw	34º18'N 78º30'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Elliptio waccamawensis*	20	1
8/10/04	Above Dam L. Waccamaw	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Elliptio waccamawensis*	10	0
8/10/04	North Shore L. Waccamaw	34º18'N 78º30'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis fullerkati*	9	2
8/10/04	Above Dam L. Waccamaw	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis fullerkati*	4	3
8/10/04	North Shore L. Waccamaw	34º18'N 78º30'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis folliculata*	1	0
8/10/04	Above Dam L. Waccamaw	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis folliculata*	2	2
4/8/05	Morrow Mnt. State Park	35º23'N 80º03'W	Stanly	Pee Dee	Yadkin-Pee Dee	*E.complanata*	15	0
4/8/05	Morrow Mnt. State Park	35º23'N 80º03'W	Stanly	Pee Dee	Yadkin-Pee Dee	*Elliptio* sp.	4	0
4/8/05	Morrow Mnt. State Park	35º23'N 80º03'W	Stanly	Pee Dee	Yadkin-Pee Dee	*Lampsilis radiata*	13	1
4/8/05	Morrow Mnt. State Park	35º23'N 80º03'W	Stanly	Pee Dee	Yadkin-Pee Dee	Pee Dee Lance	3	0

Date	Location	Latitude Longitude	County	River	Drainage	Morphological species	# Hemolymphs	# Vouchers
4/11/05	Below Lake Wacc Dam	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. fisheriana*	14	0
4/11/05	Below Lake Wacc Dam	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. folliculata*	7	0
4/11/05	Below Lake Wacc Dam	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. waccamawensis*	16	0
4/11/05	Below Lake Wacc Dam	34º15'N 78º31'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis* sp.	3	0
4/12/05	Hog Swamp	34º33'N 79º03'W	Robeson	Lumber	Yadkin-Pee Dee	*E.* sp.(green rayed)	16	2
4/12/05	Richland Swamp	34º45'N 79º10'W	Robeson	Lumber	Yadkin-Pee Dee	*E. complanata?* (unrayed periostracum)	16	3
8/2/05	Hwy 71 Bridge	34º46'N 79º20'W	Scotland/ Robeson	Lumber	Yadkin-Pee Dee	*Uniomerus carolinianus*	10	3
8/2/05	Shoe Heel Creek	34º41'N 79º23'W	Robeson	Little Pee Dee	Yadkin-Pee Dee	*E. fisheriana*	12	3
8/2/05	Shoe Heel Creek	34º41'N 79º23'W	Robeson	Little Pee Dee	Yadkin-Pee Dee	*E.* sp *(congaraea?)*	1	1
8/2/05	Shoe Heel Creek	34º41'N 79º23'W	Robeson	Little Pee Dee	Yadkin-Pee Dee	*Uniomerus carolinianus*	6	0

Date	Location	Latitude Longitude	County	River	Drainage	Morphological species	# Hemolymphs	# Vouchers
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. congaraea*	5	3
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. fisheriana*	15	3
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. folliculata*	15	3
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. icterina*	15	3
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. producta*	15	3
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*E. waccamawensis*	10	3
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Elliptio* sp.	30	5
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Elliptio* sp.(green stripes)	4	0
8/3/05	Old Dock Bridge	34º10'N 79º35'W	Columbus	Waccamaw	Yadkin-Pee Dee	*Lampsilis* sp.	4	2
Total							387	166

Appendix 3. DNA sequences obtained from GenBank

Taxon	Accession Number	Reference[1]
	16S rDNA	
Elliptio crassidens[T]	AY655034	UAUC3150
Elliptio dilatata 1	U72557	Lydeard et al. (1996)
Fusconaia flava 1[T]	AY238481	Krebs et al. (2003)
Lampsilis ovata[T]	AY655048	UAUC108
Leptodea leptodon[T]	AY655050	UAUC135
Pleurobema clava[T]	AY655060	UAUC1477
Uniomerus declivus	AY655081	UAUC3290
	Cytochrome oxidase I	
Elliptio crassidens[T]	AY613820	UAUC1493
Elliptio dilatata 1	AF231751	Bogan & Hoeh (2000)
Fusconaia flava 1[T]	AF231733	Bogan & Hoeh (2000)
Lampsilis ovata[T]	AY613826	UAUC108
Leptodea leptodon[T]	AY655003	UAUC135
Pleurobema clava[T]	AY655013	UAUC1477
Uniomerus declivus	AY613846	UAUC3290

[T] Type specimen
[1]UAUC: specimens are deposited in the University of Alabama Unionid Collection under the indicated reference number; DNA sequences in Campbell et al. (2005)