PROJECT TITLE: DNA barcode reference library for development of eDNA methods for detecting rare Arkansas freshwater mussels

PROJECT SUMMARY: The purpose of this project is to develop a DNA barcode reference library for rare and understudied mussels in Arkansas. Environmental DNA (eDNA) is rapidly emerging as a promising tool for remote detection of the presence of target organisms while at the same time, next generation sequencing techniques are making it possible to examine eDNA from entire communities through the use of DNA barcodes. For these methods to be successful, well curated reference libraries of DNA sequences from vouchered specimens are needed so that sequences obtained from environmental DNA can be accurately identified. The objective of this study is to fill in data gaps with regards to DNA sequence data for available rare and understudied freshwater mussels (Unionidae, Margaritiferidae) from Arkansas. This data will support accurate identifications based on nucleotide similarity from sequences obtained from eDNA and next generation sequencing methods.

PROJECT LEADER:

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PROJECT BUDGET:

SWG AMOUNT REQUESTED - $41,699.59

MATCH AMOUNT (35%) - $14,600.59
TOTAL AMOUNT - $56,300.18

PROJECT STATEMENT:

Background:

Freshwater mussels represent some of the most imperiled species in North America. Locating individuals and populations is a necessary first step in any conservation effort, however some species are difficult to detect, particularly when individuals are present only in low densities. Environmental DNA (eDNA) is a rapidly developing technique that can provide a means of detecting organisms without direct observation. Environmental DNA may be particularly useful where sampling is difficult or individuals are present in low densities, making them difficult to find using traditional survey methods. In some cases, eDNA has shown a higher detection rate of low density species compared to traditional survey methods (Jerde et al. 2011). Environmental DNA is present in aquatic systems in a variety of forms including recently deceased individuals, feces, sloughed epithelial cells, and gametes (Ficetola et al. 2008). Recent work indicates environmental DNA persists in aquatic environments between 6 and 30 days (Dejean et al. 2011) and persistence is dependent on the type of habitat (e.g. lotic vs. lentic). Environmental DNA is generally collected directly from water samples (Ficetola et al. 2008) or from filtration of large volumes of water (Jerde et al. 2011) and the DNA present is amplified using polymerase chain reaction. The majority of environmental DNA work to date has focused on single species surveys that utilize species-specific primers for amplifying only DNA from the target species (Ficetola et al. 2008; Jerde et al. 2011). Because of the low cost of these methods, this approach is ideal when only one species is targeted. However, designing species-specific primers may be particularly difficult if the target species occurs in a community of closely related organisms such as the case with many freshwater mussel assemblages. Recent advances in "next-generation sequencing" technologies, which involve massive parallel DNA sequencing of mixed samples, alleviate the need for species-specific primers and instead rely on universal primers for amplifying and sequencing DNA from a variety of organisms. The utility of universal primers is that it allows for analysis of entire communities from a single sample rather than targeting individual species (Meusnier et al. 2008). This approach is essentially large scale DNA barcoding (Hebert et al. 2003), and resulting sequences are compared to reference sequences to determine their identity based on nucleotide similarity. A recent study applied next generation sequencing techniques to pond water samples and identified all fish and amphibian species known from the sampling location as well as migrant species of mammals not sampled (Thomsen et al. 2011).

Need:

One current limitation to expanding the use of environmental DNA to other groups such as freshwater mussels is a lack of reference DNA sequences for accurate identification of sequences obtained from environmental DNA. While some mussel species are well represented in public DNA sequence databases (e.g. GenBank), for several species known to occur in Arkansas there are few or no reference sequences. For those species that have only one or two sequences, often they represent individuals that were collected from drainages east of the

Mississippi River and may represent genetically different lineages as is the case for many aquatic taxa. For environmental DNA sampling to be successful, DNA reference libraries are needed that include not only rare species but also representatives from, at a minimum, the regional level and ideally from the drainage level.

Fresh DNA is ideal for generating reference sequences and can be obtained from tissue clips or from non-invasive swabs. For some mussel species, populations may have declined to the point of being undetectable via traditional survey methods so obtaining fresh DNA samples using tissues or non-invasive swabs for reference sequences may be difficult or impossible. When traditional surveys fail to find fresh samples, museum specimens (preserved in ethanol or from dried adductor mussel on shells) may represent an alternative source of DNA for generating reference sequences. Older tissues obtained from museum collections are likely degraded; however, recent studies have been successful at extracting DNA from mussel specimens preserved over 50 years ago and amplifying smaller regions of DNA to combine into longer sequences (Burdick & White, 2007; K. Inoue, unpublished data).

Purpose and Objectives:

The purpose of this project is to fill in data gaps with regards to DNA reference sequences for imperiled mussels in Arkansas and make them available for comparison to sequences obtained from eDNA samples. The target species identified for reference sequence needs are those species with no or few reference sequences available in GenBank, are of conservation concern, and are known/possibly from Arkansas drainages include *Epioblasma florentina curtisi*, *E. triquetra*, *Leptodea leptodon*, *Arkansia wheeleri*, *Simpsonaias ambigua*, *Margaritifera hembli*, *Alasmidonta viridis*, *Obovaria c.f. jacksoniana* (White River drainage), *O. retusa*, *Cyprogenia aberti*, *C. stegaria*, *Cumberlandia monodonta*, *Pleurobema plenum*, and *Plethobasus cyphus* (AWAP pages 903, 906, 958, 884, 1009, 966, 874, 895, 969 and 888).

Location:

Specimens will be collected from areas with historic or recent records from drainages in Arkansas, Missouri, Kansas, and Louisiana. Additionally, specimens will be obtained from museum collections.

Approach:

One of the biggest challenges to a project such as this is in obtaining specimens or DNA samples. We propose to use a combination of field and museum surveys to collect DNA for generating reference sequences. We have identified a need for 9 collecting trips (primarily in Arkansas but also Missouri and Louisiana) to obtain fresh tissues from target species. Drainages known to historically harbor the target species include the Buffalo River, Strawberry River, Little Red River, Spring River (White River), Little River, Red River, Arkansas River, and Missouri River tributaries. To obtain additional reference sequences, we propose to survey large

museum collections that house large holdings of our target species in order to determine if suitable DNA can be obtained. These museums include the Academy of Natural Sciences in Philadelphia, Florida Museum of Natural History, Museum of Comparative Zoology at Harvard University, National Museum of Natural History in Washington D.C., and the University of Michigan Museum Of Zoology. Once samples are obtained, DNA will be extracted using standard CTAB/Chloroform methods and a 500 bp sequence of the mitochondrial gene COI will be amplified and sequenced. Sequences will be deposited in GenBank for future use by researchers utilizing eDNA methods or researchers involved in molecular systematics projects.

Expected Results and Benefits:

While it is difficult to determine how many specimens will be obtained, we estimate approximately 100 new DNA references sequences will be generated from a combination of fresh samples and museum specimens. The main benefit of this work will be that the vast majority of species known to occur in Arkansas will have at least one reference sequence available in public sequence databases such as GenBank. These sequences can then be used for confirmation of sequence identity from environmental DNA samples and can also be used for other molecular systematics studies. SGCN's that will be affected include include *Epioblasma florentina curtisi, E. triquetra, Leptodea leptodon, Arkansia wheeleri, Simpsonaias ambigua, Margaritifera hembli, Alasmodonta viridis, Obovaria c.f. jacksoniana, O. retusa, Cyprogenia aberti, C. stegaria, Cumberlandia monodonta,* (AWAP pages 903, 906, 958, 884, 1009, 966, 874, 895, 969, and 888) as well as *Cumberlandia monodonta*, *Pleurobema plenum*, and *Plethobasus cyphus.*

Literature Cited

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BUDGET BREAKDOWN (35% Match Scenario)

Proposed start date: June 30, 2013 **Completion date: June 30, 2015**

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Principal Investigator: David M. Hayes

Cost Category	Federal	Non-Federal	Total
1. Salaries and wages	$5,100 (Student worker)	$9,282 (10% PI Effort – 2 years)	$14,382
2. Fringe benefits	$87.72 (1.72% Student Rate)	$3,991.26 (43% Faculty Academic Year Rate)	$4078.98
3. Supplies	$7,000		$7,000
4. Equipment	$1,000		$1,000
5. Services and consultants	$2,000		$2,000
6. Travel	$22,721		$22,721
7. Other direct costs			
8. Total direct costs	$37,908.72	$13,273.26	$51,181.98
9. Indirect costs on Federal Request	$3,790.87 (10% limited)		$3,790.87
10. Indirect costs on Non-Federal Match		$1,327.33 (10% limited)	$1,327.33
11. Total estimated costs	$41,699.59	$14,600.59	$56,300.18

Budget Justification: A total of $5,100 is requested for a student worker, calculated at $8.50/hour for 15 hours for 40 weeks. Fringe benefits are calculated at 1.72% for graduate students for a total of $87.72. $8,000 is requested for equipment and supplies needed to for sample collection and preservation, DNA extraction, and amplification of the COI gene. $2,000 is requested for sequencing services that will be performed at Advanced Genetic Technologies Center at the University of Kentucky. A total of $22,721 is requested for travel for field collection (estimated 9 trips for a total of 25 days of travel) and for museum visitations (estimated 5 museum trips for a total of 22 days of travel) for two people. Non-federal match is provided for the 35% rate including 10% of PI salary for two years, fringe benefits calculated at 43% faculty academic year rate as well as indirect costs for a total of $14,600.59 as match. At this time, the University is able to provide a match equivalent to 35% of the requested federal funds. If a 50% match is required prior to an award being offered, the PI will work with University and third-party sources to secure the additional matching funds.

QUALIFICATIONS:

David M. Hayes: Received his PhD in Environmental Science from Arkansas State University in 2010 with an emphasis on molecular systematics of freshwater mussels and snails. He has studied the molecular ecology, evolution, and distribution of freshwater mollusks for 10 years resulting in several peer-reviewed publications. He is currently an Aquatic Invertebrate Zoologist at Eastern Kentucky University at the rank of Assistant Professor and is curator of the Branley A. Branson Museum of Zoology.

John L. Harris: Received Ph.D. in Zoology from University of Tennessee in 1986 with emphasis in taxonomy and systematics of aquatic fauna concentrating on fish and mussels. He has 30 years experience in performing mussel surveys and impact analyses resulting in numerous peer-reviewed publications and/or agency reports. He has co-directed or been a committee member for 15 graduate students researching distribution and/or life history aspects of freshwater mussels in Arkansas.