FINAL PERFORMANCE REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-147-R

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Endangered and Threatened Species Conservation

Identification of important fish hosts for East Texas freshwater mussels using genetic and ecological niche- modeling methods.

Prepared by:

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28 August 2014

FINAL REPORT

STATE: ____Texas_______________ **GRANT NUMBER:** ___TX E-147-R___

GRANT TITLE: Identification of important fish hosts for East Texas freshwater mussels using genetic and ecological niche- modeling methods.

REPORTING PERIOD: ____1 Sep 2012 to 31 Aug 2014_

OBJECTIVE(S): To generate a genetic identification key for the freshwater mussels of Texas (many of which are of conservation concern) that will be used to identify fish host species, and to identify which fish are ecologically important hosts of the mussel larvae (glochidia) using ecological niche-modeling methods.

Segment Objectives:

Task 1.– *Generation of molecular identification key for East Texas mussels and identification of fish hosts using the key.*
Task 2.– *Generation of morphological identification key for glochidia.*
Task 3.– *Refinement of existing ecological niche models.*

Significant Deviations: none.

Summary Of Progress: Please see Attachment A.

Location: Sabine, Neches, Cypress, and Sulphur Rivers in Delta, Fannin, Lamar, Red River, Bowie, Cass, Morris, Titus, Camp, Upshur, Franklin, Hopkins, Delta, Rains, Wood, Van Zandt, Smith, Henderson, Cherokee, Anderson, Houston, Trinity, Polk, Tyler, Angelina, Nacogdoches, Panola, Harrison, Gregg counties, Texas.

Cost: ___Costs were not available at time of this report, they will be available upon completion of the Final Report and conclusion of the project.__

Prepared by: _Craig Farquhar_____________ **Date:** _28 August 2014

Approved by: ______________________________ **Date:** 28 August 2014
C. Craig Farquhar

Final Report – Section 6

Title:

Identification of important fish hosts for East Texas freshwater mussels using genetic and ecological niche- modeling methods

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Reporting Period: August 2012 – August 2014

Notes on Original Tasks

Task #1. Aug 2012 – May 2013 – *Generation of molecular identification key for East Texas mussels and identification of fish hosts using the key.* Glochidia and mussels collected from fieldwork being conducted via our other Section 6 grants (Ford et al. 2010; Williams et al. 2011) will be used for sequencing and RFLP analyses. Mussels will be positively identified to species and then used to generate a molecular identification key that will then be used to identify glochidia collected from potential fish hosts.

Complete

Task #2. May 2013 – Nov 2013 – *Generation of morphological identification key for glochidia.* As glochidia are collected, morphological measurements will be taken prior to use in our molecular analyses. Once glochidia are identified to species using molecular markers, we will conduct multivariate analyses (e.g., discriminant function analyses, principle component analyses) of morphological measurements to determine if glochidia can be identified to species using only morphogical data, which will provide field researchers with a more cost and time efficient method of identifying glochidia to species.

Attempted but ultimately abandoned due to the low number of glochidia obtained for most species of mussel. Future work should concentrate on obtaining glochidia from gravid female mussels to be used in measurement trials.

Task #3. Aug 2012 – Nov 2013 – *Refinement of existing ecological niche models.* We will refine the niche models created using our current section 6 grant, by adding fish host information. This process will also identify which fish species are important for the life cycle of the mussels and which ones are incidental hosts. The niche models are based on watershed, soil, geology, and topographic GIS layers, combined with records of the locations of the mussel species in the field; we will add to the models distribution data of the possible fish hosts. The niche models are created using the MAXENT software package. MAXENT produces geospatial predictive models, which can be displayed as maps. The models show areas of suitable habitat for the focal species (mussels in this case), based on their associations with environmental variables contained in the GIS layers (Pineda and Lobo 2009, Urbina-Cardona and Flores-Villela 2010). Ecological niche-modeling has been used to model spread of invasive species (Thuiller et al. 2005), impacts of climate change (Thomas et al. 2004), and spatial patterns of diversity (Graham et al. 2006). Recent evaluations have shown MAXENT to be a robust method for modeling geographic distributions of species, even species with restricted distributions or limited records from the field, such as those of conservation interest (Ortega-Huerta and Peterson 2008; Phillips and Dudik 2008).

Complete

ABSTRACT: Six species of freshwater mussel are of conservation concern throughout their range in East Texas, (Texas pigtoe (*Fusconaia askewi*), triangle pigtoe (*Fusconaia lananensis*), southern hickorynut (*Obovaria jacksoniana*), sandbank pocketbook (*Lampsilis satura*), Louisiana pigtoe (*Pleurobema riddellii*), and Texas heelsplitter (*Potamilus amphichaenus*)). These species warranted listing in Texas due, in part, to their restricted distributions and low abundances. These mussels, like most unionids, exhibit an unusual life cycle, unique to Unionidae, in which their larvae, called glochidia, are obligate ectoparasites on fish. Knowledge of host fish species is severally lacking for many mussels, as many hosts are unknown or have not been verified. Such natural history data is critical to the conservation of unionids.

The purpose of this study was to evaluate if host fish identified in the laboratory act as hosts in natural populations. In addition, new species will be assessed as possible hosts by sampling naturally parasitized fish in the wild. Morphological identification of glochidia to the species level is very difficult due to the small size of glochidia (50-500 µm), therefore a species molecular identification dataset utilizing the sequence of the ND1 gene was developed prior to sampling naturally parasitized fish.

A molecular identification dataset designed from sequences of 37 mussel species found in East Texas was successfully designed and utilized to identify encysted glochidia on wild-caught fish. A total of 151 glochidia were successfully identified from eight mussel species. New potential fish hosts were identified for two state-threatened species, *Fusconaia askewi* (Texas pigtoe) and *Pleurobema riddellii* (Louisiana pigtoe). Glochidia abundance and diversity was found to differ over the sampling season within the Sabine River. Ecological niche modeling in Maxent supported the results found in fish host use of naturally encysted glochidia. These

findings are critical for understanding the complex relationships between mussels and their fish hosts, which is necessary for conservation planning.

The freshwater mussel fauna of North America is the richest of any continent, but over 70% of these organisms are listed as endangered, threatened, or species of special concern (Williams et al., 1993). The imperilment of this group is strongly influenced by anthropogenic factors including habitat degradation, construction of dams, and introduction of exotic species, such as bivalves and fish (Layzer et al., 1993; McMurray et al., 1999; Haag, 2012). Complicating the management of this group even further is the fact that life-history information is severally lacking for many species, as this information is critical in the conservation and recovery of freshwater mussels.

An important life-history trait unique to many freshwater mussels, is their unusual lifecycle, in which their larvae (glochidia) are obligate ectoparasites on fish hosts. Glochidia can only transform into juveniles on suitable host fish (Neves et al., 1985). A common problem with the conservation of many imperiled species is the lack of host fish knowledge. The co-evolutionary relationship between mussels and their host fish creates complications on the ability of mussels to reproduce successfully. Fragmentation and habitat modification caused by dams inhibit fish movement, alter the fish community, and can ultimately displace essential host fish required for mussel recruitment. The introduction of exotic fish species can negatively impact unionids through unsuccessful infestations on non-suitable hosts. Therefore, a complete understanding of fish host use is needed for an effective conservation plan.

Only a few studies have attempted to examine host use by sampling naturally parasitized fish and identifying the encysted glochidia through morphological features. When only one or a few mussel species are present in a locality, successful identification of glochidia with morphological characters has been possible (Stern and Felder, 1978; Trdan and Hoeh, 1982; Jansen, 1990; Hastie and Young, 2001; Martel and Lauzon-Guay, 2005). However, in more

species rich communities, more complications arise. Glochidia are usually not easily identified below the genus or subfamily level (Wiles, 1975; Zale and Neves, 1982; Bruenderman and Neves, 1993; Weiss and Layzer, 1995), and misidentification of glochidia can also occur (Hoggarth, 1992). Glochidia sizes range from 50 to 500 µm, and small differences in morphological characteristics are difficult to detect for species identification.

Recently some works have attempted to create molecular identification keys to counter the difficulties in morphological identification of glochidia (White et al. 1996, Gerke and Tiedemann, 2001; Gustafson and Iwamoto, 2005; Kneeland and Rhymer, 2007). Although these keys have been useful, they rely on restriction fragment length polymorphisms (RFLPs) for identification, which can have some drawbacks. Creating keys for species-rich areas, such as East Texas, can be very problematic because identifying unique digestion patterns for each species present can be difficult when closely related species share the same geographic range. Also, a different key must be created for each study area because of differences in species assemblage. A key created for mussel species of East Texas will be of little use elsewhere. Therefore, a more appropriate approach for the identification of naturally parasitized glochidia, is to use a sequence-based DNA barcoding dataset. Boyer at al. (2011) used a DNA barcoding dataset to successfully identify naturally parasitized glochidia from a very diverse mussel bed.

The purpose of this study was to determine host fish for the six state-threatened mussel species located in East Texas by sampling naturally parasitized fish throughout the mussels' known ranges. A molecular identification dataset was used to aid in identification. The goal was to not only examine previously identified fish hosts in natural conditions, but also to determine if additional hosts are used by East Texas mussels. Fish were sampled at two sites from the Sabine River and Neches River drainages during the entire known gravid periods of mussels in the area.

Glochidia encysted on gills were identified with the molecular identification dataset. Spatial differences in host use between the two rivers and any temporal differences in host use between sampling dates were also examined.

MATERIALS AND METHODS

Tissue Sampling

Specimens for the 37 mussel species from the Neches River drainage and Red River drainage were previously collected for mussel survey studies in East Texas (Ford, 2013) and deposited in the Ford lab at the University of Texas at Tyler. Mussels were located by timed searches along the banks of rivers and streams. The adductor muscles of each specimen were cut to prevent the mussel from closing shut, and then placed in 95% ethanol and returned to the laboratory. Mussels were cleaned and approximately 15mg of tissue from the ventral margin of the mantle and the adductor muscles was kept to ensure there was adequate tissue for DNA sequencing.

DNA Sequencing

DNA was extracted from adult mussel tissue using an Illustra tissue and cells genomicPrep mini spin kit (GE Healthcare, Buckinhamshire, UK) following the manufacturers protocol. Genomic DNA was then resuspended in 100 µL of elution buffer and stored at -20° C until use in polymerase chain reactions (PCRs). Amplification of the mitochondrial (mtDNA) NADH dehydrogenase (ND1) gene was carried out using the primers Leu-uurF and LoGlyR (Serb et al. 2003). Twenty µL PCR reactions were used for amplification and consisted of 7.1 µL H2O, 2.0 µL TopTaq PCR buffer (Qiagen), 0.4 µL dNTPs, 2.0 µL Coral Load (Qiagen), 4.0 µL Q-solution, 1.0 µL each 2- µmol primer, and 2.4 µL DNA (~200ng). A negative control was included with each PCR. Reactions were amplified with an Eppendorf Mastercycler gradient

thermal cycler with a temperature controlled lid. Reaction conditions for double-stranded amplification consisted of an initial denaturation at 94° C for 5m, followed by 30 cycles of 94° C for 45 s, 54° C for 60 s, and 72° C for 60 s, and a final extension of 72° C for 5m. PCR products were purified using an E.Z.N.A. cycle pure kit (Omega bio-tek, Norcross, GA) following the manufactures protocol and resuspended in 60 µL of sterile water. Purified DNA was concentrated to the level recommended by Eurofins MWG Operon (20–40ng/µL) and shipped to Eurofins MWG Operon for sequencing reactions using BigDye Terminator v 3.1 Cycle Sequencing kits (Applied Biosystems).

Glochidia were processed with similar methods except for a few minor modifications. For example, genomic DNA was extracted from a single glochidium using an Illustra tissue and cells genomicPrep mini spin kit (GE Healthcare, Buckinhamshire, UK) with a slightly modified spin procedure. Specifically, the amount of buffers and proteinase K used in each step was reduced by one half to avoid diluting of the genomic DNA (Kneeland and Rhymer 2006). It was then resuspended in 75 µL of elution buffer. The PCR mixture and thermocycler settings remained unchanged. Because of unwanted interference of fish DNA, a nested PCR approach was used for clear amplification of glochidia DNA. The initial amplification was done using the primer pair Leu-uurF and LoGlyR (Serb et al., 2003), and then followed with a second amplification using Leu-uurF and NIJ. Purification and sequencing followed the same protocol as the adult mussels.

<u>*Development of ND1 Dataset*</u>

Fifty-eight sequences from mussel tissue collected in East Texas were combined with 122 sequences available on the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov), for a total of 180 sequences within the 37 mussel species located

in East Texas. These sequences were used to develop the molecular identification dataset to easily identify naturally encysted glochidia.

Field Sites

Fish sampling was conducted at one site at the Sabine River (HWY 14) and one site at the Neches River (HWY 294). Sites were specifically selected where previous mussel surveys exhibited a high abundance and diversity of state-threatened species. The Sabine site, for example, is inhabited by at least 13 species of freshwater mussel, including three state-threatened species (i.e., the Texas pigtoe, sandbank pocketbook, and Texas heelsplitter) found throughout the Sabine River drainage. The Neches River site was chosen to increase the probability of finding all six of the state-threatened species, which are all present within the Neches River drainage. The continual sampling at one primary site is useful for determining true host infestations compared to accidental infestations that would eventually slough off fish. It is also useful for determining spawning times for mussel species lacking life-history information.

Sampling Methods

The Sabine River site was sampled bimonthly from March to August of 2013. There is vast variability in spawning time between freshwater mussel species with some species becoming gravid early in the spring when the water begins to warm, while other species may not become gravid until late summer/early fall (Haag, 2012). Gravid Texas pigtoes, triangle pigtoes, and Texas heelsplitters have been observed in July; gravid southern hickorynuts have been observed in late summer; gravid sandbank pocketbooks and Louisiana pigtoes are unreported (Howells et al., 1997). Fish were sampled from the Sabine throughout the spring and summer months in an attempt to encompass all of the gravid months for each target species. Sampling dates were spread relatively evenly in the Sabine River, (~ every 2 weeks), to examine for

temporal variation in host use. The Neches River site was only sampled once in June and once in August during the start and end of the mussel spawning season in an attempt to widen the diversity of glochidia captured.

At both sampling sites, fish were captured at a large diverse mussel bed using two sampling methods: beach seining and electrofishing. Sites were composed of many different habitats, such as riffles and pools, to increase the diversity of not only fish species captured but also mussel species present. Standardized sampling conditions were implemented in an effort to catch the same amount of fish each sampling date. The conditions consisted of a three man sampling team, in a 150m river reach, for one hour on each date. Fish were sampled from a variety of species and sizes, to examine for fish hosts with varying microhabitat use and behavioral characteristics. A bag seine measuring 7.5m long was used throughout the river sites to capture sunfish, minnows, darters, and juveniles/young of the year of various species. A Halltech Aquatic Research INC. HT-2000 backpack electrofisher followed by the bag seine was used throughout the entire river to optimize the diversity of fish species captured.

Fish species were identified in the field, sacrificed with a lethal dose of tricaine methanesulfonate (MS-222; 200mg/L), and preserved in 95% ethanol. Because fish larger than 15cm were too large to return to the laboratory, such specimens received a lethal dose of MS-222 and their gills and fins were subsequently removed in the field to be processed later. An attempt was made to visualize encysted glochidia in the field but was unsuccessful; therefore, all fish were kept for processing to ensure accurate infestation rates. All fish were examined to investigate host effectiveness for each species. Fish were returned to the lab for examination of gills and fins under either a compound light microscope or a dissecting microscope. Fins were cut from fish and examined under a dissecting microscope. Gills were excised from fish and

examined for glochidia under a compound light microscope with the addition of 2% KOH, which aided in the visualization of encysted glochidia. Individual glochidia were removed with dissecting probes, and an effort was made to minimize the amount of gill tissue attached to the glochidia. Individual glochidia were preserved in 95% ethanol and kept at -20° C until needed for DNA extractions.

Initial Maxent Models

Previous niche models for six state-threatened mussel species (Texas pigtoe (*Fusconaia askewi*), triangle pigtoe (*Fusconaia lananensis*), southern hickorynut (*Obovaria jacksoniana*), sandbank pocketbook (*Lampsilis satura*), Louisiana pigtoe (*Pleurobema riddellii*), and Texas heelsplitter (*Potamilus amphichaenus*)) were used as the initial models for this study (Walters et al. in print). The models were based on the known locations of the species and six environmental layers: soil type, geology, vegetation type, landform, groundwater recharge, and land cover type.

Creation of Fish Layers

Extensive fish surveys were conducted from 2009-2012 in East Texas. Twenty-eight sites within the Angelina, Neches, Sabine, and Sulphur Rivers were surveyed (Troia, 2010; Dunithan, 2012). This species location data was used to create individual fish species distribution models in Maxent. The same six environmental layers used to model the previous mussel distributions were used to model the fish distributions. A species' distribution was only modeled if more than five recent records were available for that fish species (Pearson et al., 2007).

Mussel Models Updated with Data on Potential Fish Hosts

The distributions of the fish species were used as new environmental layers to rerun the previous distribution models for the mussels. In total 23 fish layers (corresponding to the forecast distributions of 23 different fish species) were incorporated as new data into the new mussel models (Table 3). These new mussel models were run according to Walters et al. 2014. The mussel models were evaluated using AUC and the deviance (measured in Maxent by test gain; Phillips and Dudik, 2008). AUC is useful in comparing different models to one another and in deciding if a model is "accurate enough," but it does not assess the goodness-of-fit of the model (Lobo et al., 2007). To determine the relative importance of different environmental components (i.e., the layers) to the model for a specific mussel species, the gain of the overall model was compared to the gains of the specific environmental components when modeled alone. The jackknifing option in Maxent was used to calculate the average effect on the total model fit of (a) leaving each environmental component out of the mussel model, in turn and (b) leaving out all other environmental components except for the target, in turn. These complementary approaches allow one to evaluate (a) how much of the total model fit (gain) can be explained by an individual environmental component and (b) how much of the information provided by an environmental component is unique, i.e., not already provided by another environmental component in the model.

RESULTS

Development of Molecular Dataset

Alignment of the ND1 gene yielded 950 bp of usable sequence for interspecific and intraspecific comparisons. Intraspecific variation was low for all species. There were only four variable sites (0.4% divergence) among *F. askewi* sequences, four variable sites (0.4%

divergence) among *F. lananensis* sequences, and zero variable sites between pairs of *O. jacksoniana* and *P. amphichaenus*. The number of variable sites among the sequences for the other 31 mussel species in East Texas ranged from zero in *Lasmigona complanata* to 17 (1.7% divergence) in *Pyganodon grandis.*

Interspecific variation was high between most species, allowing for easy species identification with just one gene. For example, two closely related species that are thought to hybridize, (i.e. *P. amphichaenus* and *P. ohiensis*) only had an 86% identity score for the ND1 gene. However, the variation in ND1 between *F. askewi* and *F. lananensis* was < 1.0%, causing problems in separating the two species. Other genes were examined for greater variation between the two species (COI and ITS), but also were too low to successfully differentiate the two (Burlakova, 2012). Although the two species cannot be separately distinguished with analysis of various gene sequences, they do not co-occur in the same localities and even differ among the river drainages in which they are distributed (Ford, 2012).

<u>*Prevalence and Abundance of Glochidia Infestation on Wild-Caught Fish*</u>

A total of 1566 fish representing 43 species were captured and examined for the presence of glochidia from the Sabine River, whereas 142 fish representing 13 species were collected and examined from the Neches River. Based on laboratory examination, 578 fish (37%) representing 23 species were infested with one or more glochidia from the Sabine River (Table 1); and 87 fish (61%) representing 7 species were infested with one or more glochidia from the Neches River (Table 2). Infestation prevalence and intensity were highly variable among species and ranged from 0% to 100%. Among species captured from the Sabine River most often (≥20 fish examined), blacktail shiner (*Cyprinella venusta*) was parasitized most frequently (72%) and was often heavily parasitized (18% with ≥20 encysted glochidia). Red shiner (*Cyprinella lutrensis*)

was similarly infested (64%), but tended to be more heavily parasitized (22% with ≥20 encysted glochidia). Among the remaining species where ≥20 individuals were examined, infestation rates ranged from 8% in the western mosquito fish (*Gambusia affinis*) to 36% in dusky darters (*Percina sciera*). Excluding blacktail shiners and red shiners, only 3 of the remaining 142 fish were heavily infested with glochidia. Similar infestation rates were seen in red shiners and blacktail shiners from the Neches River (81% and 75% respectively). Glochidia were mostly encysted on fish from the family Cyprinidae, where red shiners, blacktail shiners, and bullhead minnows were infested with 96% of the glochidia examined (n=7266).

For the entire sampling period in the Sabine River a total of 6721 glochidia were found. The Sabine River sampling dates were compared to obtain a better understanding of variation in glochidia abundance and host use (Figure 1). The greatest glochidia abundance throughout the field season occurred in two large spikes on three sampling dates. The first spike occurred on May 14th, where 1709 (25% of total captured glochidia) were found. The glochidia captured fell by a third for the next sampling date. The second spike occurred on June 11th and continued through June 25th. The second spike lasted through two sampling dates and totaled 2664 (30%) captured glochidia. The other nine sampling dates examined had significantly less glochidia and ranged from 29 (0.4%) to 695 (10%) glochidia. The highest percent of infested fish and largest number of infested fish species occurred on the three dates where the greatest number of glochidia were found.

Eight hundred and twenty-four glochidia were captured for two sampling dates in the Neches River. These two dates occurred close to two dates from the Sabine River, and these data can be compared for any spatial variation in glochidia abundance or host use. The Neches River was found to have lower abundance of glochidia on both sampling dates when compared

with the Sabine River, however, both rivers followed a similar trend. The early June sampling dates yielded significantly greater numbers of glochidia than the August sampling dates (Neches River 767:57, Sabine River 1376:187).

<u>Laboratory Identification of Glochidia</u>

Considering 7545 glochidia were recovered over the sampling period there was not enough resources to sequence each individual glochidia. Therefore, glochidia were systematically grouped by four categories. They were first split by the river drainage in which the fish were captured (Sabine vs. Neches). Then they were divided by the sampling date in which they were found to account for temporal changes in host use. To investigate each potential species of host fish, glochidia were then grouped by the fish species they were encysted on. Lastly, glochidia were grouped into size and shape classes in an effort to sample every mussel species captured. Multiple glochidia were randomly selected from each group for sequencing. When fish were infested with more than one glochidia that were all morphologically similar, only one was chosen for sequencing. As a result 151 out of 7545 glochidia were successfully sequenced from 20 of 23 infested species. DNA could not be extracted and amplified for sequence data from glochidia attached to brook silverside (*Labidesthes sicculus*), pirate perch (*Aphredoderus sayanus*), or slough darter (*Etheostoma gracile*).

A total of 190 glochidia were processed for sequencing and 151 (79%) were successfully amplified and identified to species. From the Sabine River, *F. askewi*, one of the species of interest, was by far the most common species identified, comprising about 87% (n=104) of identified glochidia. Five other species were identified from encysted glochidia in the Sabine River. *Fusconaia askewi* also comprised the majority of the glochidia identified from the

Neches River (68%, n=21). Most importantly, *P. riddellii* glochidia, another target species, was also successfully identified in samples collected from the Neches River (19%, n=5). No other state-threatened mussel species were identified from the remaining glochidia.

Potential New Host Fish for F. askewi

No previous studies have investigated the potential fish hosts used by *F. askewi*. One hundred and four *F. askewi* glochidia were successfully identified on 17 of the 23 fish species infested from the Sabine River. Because of the close relationship, these glochidia were identified as either *F. askewi* or *F. lananensis* with the genetic dataset. However, only *F. askewi* is located within the Sabine River drainage. Therefore, with the genetic sequence and locality of sampling, the species of mussel was easily determined. Most fish were infested with more than one glochidia, and these fish most likely carried many more *F. askewi* glochidia than were processed for identification. The majority of the *F. askewi* were encysted on red shiners (49%, n=52). Of the 17 fish species, only four had greater than five identified *F. askewi* glochidia (red shiner, blacktail shiner, bullhead minnow, and longear sunfish). *Fusconaia askewi* glochidia were found throughout the entire sampling season starting in March and ending in August. *Fusconaia askewi* glochidia host use was evenly distributed by sampling date, with an average of five host fish bimonthly.

A *Fusconaia* species was also identified from 21 glochidia encysted on fish gills from the Neches River. These glochidia were identified as either *F. askewi* or *F. lananensis* with the molecular identification dataset, and both species are present throughout the Neches River drainage. However, the two species do not co-occur and extensive surveys have revealed an abundant *F. askewi* mussel bed at the sampling site (Ford, 2013). These glochidia were identified on four fish species, all of which were also infested with *F. askewi* in the Sabine River.

The sampling dates in June and August from the Neches River both had successfully identified glochidia as *Fusconaia.*

Potential New Host Fish for P. riddellii

No previous studies have investigated the potential fish hosts used by *P. riddellii*. Five glochidia from the Neches River on June 5th, 2013 were successfully identified as *P. riddellii.* These glochidia were encysted on the gills of three red shiners and one bullhead minnow. Most fish were infested with more than one glochidia, and it is likely that most fish, if not all, carried many more *P. riddellii* glochidia than were processed for identification. No *P. riddellii* were identified from the Sabine River or the August sampling date in the Neches River.

Confirmed and Potential New Host Fish for the Remaining Species Identified

New potential hosts were identified for *Amblema plicata* (threeridge). Glochidia were identified on fish from the Neches River during initial sampling on June 5th, 2013. Five *A. plicata* glochidia were identified on three potential new hosts (red shiner, blacktail shiner, and longear sunfish). No previously laboratory identified host fish were encysted with *A. plicata* glochidia. Red shiner had the greatest number of encysted *A. plicata,* (n=3), while blacktail shiner and longear sunfish each only had one fish infested. Although only five glochidia were identified, each of the five infested fish were heavily infested and possibly carried more *A. implicata* glochidia.

No previous studies have identified any potential fish hosts used for *Quadrula mortoni* (western pimpleback). Glochidia were identified as *Q. mortoni* on five fish species from the Sabine River during only one of the sampling periods (June 14th). Only one glochidia was identified as *Q. mortoni* on each of the five fish species infested. All five fish had low levels of infestation, with less than three encysted glochidia. The spotted bass infested with a *Q. mortoni*

was also infested with *F. askewi*. *Quadrula mortoni* was the only glochidia identified on largemouth bass (*Micropterus salmoides*) during the entire sampling period.

Previous studies have identified *Quadrula verrucosa* (pistolgrip) to be capable of metamorphosis on yellow bullhead (*Ameiurus natalis*) and on flathead catfish (*Pylodictus olivaris*) (Hove et al., 2004). Although flathead catfish were captured in this study, none were infested with glochidia. However, three new potential hosts were identified for *Q. verrucosa*. Two glochidia from the Sabine River during the March 8th sampling were identified as *Q. verrucosa*. These glochidia were encysted on a mud darter (*Etheostoma asprigene*) and a weed shiner (*Notropis texanus*). One glochidia was also identified from the Sabine River during the April 9th sampling. This glochidia was encysted on a western mosquito fish (*Gambusia affinis*). *Q. verrucosa* was the only glochidia identified on mud darters. All three of these fish encysted with *Q. verrucosa* were infested with less than two glochidia.

Previous laboratory studies have been unsuccessful in identifying potential fish hosts used for *Plectomerus dombeyanus* (bankclimber). Glochidia were identified as *P. dombeyanus* on four fish within two species from the Sabine River during two sampling periods (June 25th and July 8th). Three of these glochidia where encysted on red shiners while one was encysted on a blackstripe topminnow (*Fundulus notatus*). Two of the red shiners were heavily infested with glochidia, but they were also encysted with *F. askewi*. Based on morphological features, only one *P. dombeyanus* was encysted on each of the four fish.

Only one host species has been identified through laboratory studies for *Potamilus purpuratus* (bleufer) and it was not confirmed in this study. However, a new potential host was found. *Potamilus purpuratus* glochidia were successfully identified on one fish from the Sabine River during one sampling period (June 11th). One red shiner was found to be encysted with

three *P. purpuratus* glochidia. This red shiner was heavily infested and was also infested with *F. askewi.* The *Potamilus* glochidia are easily identified based on their axe shape and no other *Potamilus* were morphologically found throughout the rest of the study.

One *Truncilla truncata* (deertoe) glochidia was identified on a freshwater drum (*Aplodinotus grunniens*) from the Sabine River during one sampling date (June 28th). This fish has shown to be a potential host for *T. truncata* in previous studies. Other freshwater drum were infested with glochidia throughout the sampling period but only one was able to be identified. This freshwater drum had only the one glochidia encysted on its' gills. *Truncilla truncata* was the only glochidia encysted on freshwater drum during the entire sampling period.

<u>Ecological Niche Modeling</u>

Twenty-three fish species were successfully modeled with AUC values >0.75. These 23 models were considered good at predicting suitable habitat for these fish species, and thus were used as layers in the creation of updated mussel models. Niche models for 7 of the 23 fish species found to be encysted with glochidia in the wild were unable to be created into environmental layers, as there were either too few location sites (<5), or they were insufficient models (AUC <0.75). However, these seven species all had less than three encysted glochidia found throughout the entire sampling season. These infestations might have been the result of accidental attachment, and not the result of a true host fish interaction. Models were successfully created for all of the heavily infested fish species. Also, fish models were successfully created for the two fish species encysted with *Pleurobema riddellii* glochidia, and for 12 of the 17 fish species encysted with *Fusconaia askewi* glochidia.

Potential Fish Host

The updated mussel models that included the fish distribution data all had AUC values > 0.9. Because AUC is not a measure of model fit (Lobo et al. 2007), the AUC values of the new mussel models were not compared to the AUC values of the old models from Walters et al. (2014). Instead, the focus was on dissecting the contributions of individual environmental components to the new mussel models, specifically concentrating on how much the fish distributions contributed to the gains of the individual mussel models and how much unique information the fish distributions added to the individual mussel models.

For *P. riddellii*, there were two fish species (blacktail shiner (*Cyprinella venusta*) and dusky darter (*Percina sciera*)) whose distributions when modeled alone were each able to account for 75% of the total gain of the full mussel model that included all environmental variables (Table 4). For the *Obavaria jacksoniana*, there were five fish species (blackstripe topminnow (*Fundulus notatus*), blacktail shiner (*Cyprinella venusta*), dusky darter (*Percina sciera*), red shiner (*Cyprinella lutrensis*), and weed shiner (*Notropis texanus*)) whose distributions when modeled alone were each able to account for >50% of the total gain of the full mussel model that included all environmental variables (Table 4). For the *Potamilus amphichaenus*, there were nine fish species (bluegill (*Lepomis macrochirus*), bullhead minnow (*Pimephales vigilax*), dollar sunfish (*Lepomis marginatus*), dusky darter, freckled madtom (*Noturus nocturnus*), longear sunfish (*Lepomis megalotis*), red shiner, smallmouth buffalo (*Ictiobus bubalus*), and spotted bass (*Micropterus punctulatus*)) whose distributions when modeled alone were each able to account for 90% of the total gain of the full mussel model that included all environmental variables (Table 4). For the *F. askewi*, there were 21 fish species (blackstripe topminnow, blacktail shiner, bluegill, bullhead minnow, channel catfish (*Ictalurus*

punctatus), dollar sunfish, dusky darter, freckled madtom, freshwater drum (*Aplodinotus grunniens*), gizzard shad (*Dorosoma cepedianum*), largemouth bass (*Micropterus salmoides*), longear sunfish, orangespotted sunfish (*Lepomis humilis*), pirate perch (*Aphredoderus sayanus*), red shiner, ribbon shiner (*Lythrurus fumeus*), smallmouth buffalo, spotted bass, weed shiner, western mosquito fish (*Gambusia affinis*), and white crappie (*Pomoxis annularis*)) whose distributions when modeled alone were each able to account for a substantial portion of the total gain of the full mussel model that included all environmental variables, especially the cyprinid and centrarchid species (Table 4). The fish distributions were poorer at accounting for the full model fit in the case of the *F. lananensis* mussel model (Table 4).

DISCUSSION

Use of Molecular Dataset

This dataset was shown to be very accurate at distinguishing between species of unionid mussels. Misidentifying a species could not only provide false information on host status for a mussel species, but it could also alter future laboratory studies, leading to future studies examining wrong hosts for metamorphosis suitability. A recent study (Boyer et al., 2011) using a similar approach determined that both CO1 and ND1 loci perform well for species identification but that the barcoding gap between average intra- and interspecific genetic distances is wider for ND1. Their unionid dataset showed nearly double the interspecific variation with similar intraspecific variation when comparing ND1 to COI. For this reason, ND1 appears to be the more appropriate locus to use for a DNA barcoding dataset designed for unionid mussels, which is why it was utilized here.

By using a DNA barcoding approach with the ND1 gene, species can be easily identified by the analysis of a single gene sequence, with the exception of the *F. askewi* and *F. lananensis*.

This low interspecific variation could reflect very recent divergence of the two species, mitochondrial introgression caused by hybridization, or as suggested by Burlakova (2012), the incorrect splitting of one true species. Considering the low variation in the ND1 gene between the two species, RFLP analysis would also be inefficient in separating the species, because RFLP's utilize interspecific differences. This key, however, is still useful for the identification of naturally infested glochidia, because of the fact that these two species do not co-occur. One can infer which species of *Fusconaia* a particular glochidia is based on the river drainage and specific site in which the naturally infested fish was captured.

An effective molecular identification dataset will only be accurate if DNA is successfully extracted and amplified from a single glochidium. The extraction methods demonstrated in this study successfully amplified the small amount of DNA available in one glochidium. While the extraction process did not work on 100% of the glochidia (151 out of 190), it did work on a high enough portion to be a suitable method for identification.

Previous studies have developed identification keys utilizing the techniques of RFLP analysis (White et al., 1996; Gerke and Tiedmann, 2001; Kneeland and Rhymer, 2007). The use of DNA barcoding approach developed in this study is significantly easier to use. After amplification is completed, sequencing of the ~1000 bp region yields enough variation to separate species, much easier than the multistep restriction enzyme procedure applied for unionids in Maine (Kneealnd and Rhymer, 2007). The simplicity of DNA barcoding demonstrates how increased availability of genetic sequence data and new advancing tools can easily be utilized to create an incredibly accurate molecular identification dataset. This method will still be useful in more complex ecosystems with higher diversity than 37 mussel species.

Molecular identification of glochidia through the use of a molecular key has many advantages over traditional laboratory trials. Host effectiveness with laboratory methods can be measured based on encystment time and the number of metamorphosed juveniles. Whereas, naturally parasitized fish can provide a more natural insight of host effectiveness by quantifying both the proportion of fish captured with glochidia infestations and the infestation intensity, which may differ among fish species and fish age (Martel and Lauzon-Guay, 2005). Sampling naturally parasitized fish also has the advantage of examining a larger number of fish species. Within the study area, any fish species captured can be examined for encysted glochidia, compared to the limited resource availability in laboratory studies determining the number of species tested. However, a natural study will be limited on the number of species examined based on capture techniques used. Capture techniques can be useful in targeting various age classes and sizes to examine host use in each stage of the fish's life history. Laboratory studies are usually constrained to examining hosts for one or only a few mussel species, but when utilizing molecular identification techniques, sampling naturally parasitized fish could yield information on host use for all of the mussel species found in a geographical area. The ideal approach to determining host use and host effectiveness for a particular species of mussel involves a combination of field sampling with molecular identification of glochidia from naturally infested fish, paired with laboratory studies to confirm successful juvenile metamorphosis. This approach allows for laboratory trials to be focused on fish species found as potential hosts in the natural settings, opposed to examining fish species based on hypotheses that may not have any significance in natural populations.

Host Effectiveness of Confirmed and Potential Host Fish

The prevalence and abundance of *F. askewi* and *P. riddellii* on naturally parasitized fish indicates that not only do some cyprinids act as suitable hosts in the wild, but they are likely the most effective host contributing to recruitment of both mussel species. *Fusconaia askewi* was found on 57 of the 65 (88%) red shiners, 16 out of 17 (94%) blacktail shiners, and 13 out of 14 (93%) bullhead minnows examined for identification of glochidia from both the Sabine River and Neches River throughout the entire field season. Other fish were infested with *F. askewi* but not at the high rates seen in the cyprinid species sampled. *Pleurobema riddellii* was found on 3 out of 10 (30%) red shiners, and 1 out of 2 (50%) bullhead minnows examined for identification of glochidia from the Neches River during the June 5th sampling. Had each glochidium been identified, it is likely that many more *F. askewi* and *P. riddellii* would have been identified on these cyprinid species. All three of these minnow species had high infestation rates and were often heavily infested. Other cyprinids were captured but not all were infested with glochidia.

In addition to the highly infested cyprinids, *F. askewi* was encysted on four other minnow species, pallid shiner (*Hybopsis amnis*), golden shiner (*Notemigonus crysoleucas*), weed shiner (*Notropis texanus*), and fathead minnow (*Pimephales promelas*). These four species had much lower infestation rates and are probably less ecologically important hosts in the wild. In total, *F. askewi* was identified on 17 of the 23 infested fish, within eight families, and five of these fish species were encysted with greater than five *F. askewi.* Other *Fusconaia* species have shown to have selective host use on many minnow species (Neves, 1991; Haag and Warren, 2003; Williams et al., 2008), and similar trends were seen in this study, with high infestation rates on three cyprinid species. However, considering the wide range of fish families infested with *F. askewi*, this species is probably more of a generalist than other closely related *Fusconaia*. Red

shiners, blacktail shiners, bullhead minnows, and longear sunfish carried the most *F. askewi* and were also the only fish species to be highly infested (≥20 glochidia). This trend indicates that these four species are the most effective host for *F. askewi* in the wild.

Only five *P. riddellii* were identified throughout this study. These glochidia were found on only four fish and each fish had less than three encysted glochidia. Whereas *F. askewi* was usually heavily infested on fish gills, *P. riddellii* appears to be in lower abundances when encysted on fish. Similar to this study, other *Pleurobema* species have been known to use minnows as host fish (Hove and Neves, 1994; Gibson et al., 2011). Considering *P. riddellii* was only found to infest two species, red shiner and bullhead minnow, these two hosts probably play an important ecological role in the distribution of this mussel species.

Considering unsuitable fish species are capable of carrying glochidia for a few days prior to an immunological response (Meyers et al., 1980; Watters and O'Dee, 1996), laboratory trials must be conducted with these potential host species to confirm that metamorphosis into juveniles occurs. However, the formation of a cyst has been suggested as evidence that successful metamorphosis will ocurr (Martel and Lauzon-Guay, 2005). Knowledge of host status for these species would provide conservation management strategy for both mussel species. With the exception of the four highly infested fish species, the other potential new host fish may not contribute significantly to recruitment in East Texas considering only a very small percentage of these fish were found with *F. askewi* glochidia. Although, a host's effectiveness is also dependent on abundance of these fish, which may lead to an underestimation of importance based on infestation rates alone.

Life-history of Confirmed and Potential Host Fish

An effective host fish will exhibit the necessary life-history characteristics for attachment and transformation of glochidia. Not only must the range of the host fish overlap with the mussel species, its' behavior must be favorable in bringing the fish into close proximity with a gravid mussel. Many Ambleminae species, such as *F. askewi* and *P. riddellii*, have evolved elaborate conglutinate structures, which can mimic minnows or invertebrates, to attract host fish (Haag and Warren, 1999). Other species that lack a mantle lure may use temperature or photoperiod mechanisms to effectively release glochidia nearby the appropriate fish host (Haag, 2012). Some species even respond to tactile stimulation or through chemical cues to detect the presence of potential fish host (Bauer, 2001; Henley and Neves, 2001).

The cyprinid species encysted with glochidia in this study are most likely a large factor in the dispersal of these threatened mussel species. However, it is not certain if current populations are actively dispersing glochidia, and thus dictating the distribution of these mussels. These minnows species are typically among the most abundant fish species in East Texas river drainages (Marsh-Matthews and Matthews, 2000), and were universal at the two study sites. The three highly infested minnow species are omnivores (Hale, 1963; Laser and Carlander, 1971), and large individuals are likely attracted to free-floating pelagic conglutinates released by these two mussel species.

Red shiners, in particular, have life-history traits that suggest this species could be a vital host in recruitment, if a mussel was capable of metamorphosis. Red shiners are extremely tolerant of harsh physical conditions, including temperature and oxygen stress (Matthews and Maness, 1979; Matthews, 1987). The species is a habitat generalist (Douglas et al., 1994), readily invades rewatered habitats (Matthews, 1987; Cross and Collins, 1995), can reproduce in

its first summer of life (Marsh-Matthews et al., 2002), and is highly invasive outside its native range (Olden and Poff, 2005), all factors that would aid in the dispersal of freshwater mussels.

The dispersal of most unionid species is strictly dependent upon the fish hosts in which they infest (Watters, 1992), and it is thought that the distribution of a mussel species is heavily influenced by the effectiveness and breadth of host fish utilized. However, the high infestation rates and wide range of host use seen in *F. askewi* would suggest that other factors are more crucial in their distribution. *Pleurobema riddellii*, on the other hand, had low prevalence and was only encysted on two fish species, within the Cyprinidae family. This could indicate that the conservation status of *P. riddellii* is strongly influenced by the ability of this mussel species to successfully encounter and attach a suitable host fish. This information will be vital knowledge in the conservation efforts to restore threatened mussel populations.

Only four fish species were examined for a trend in infestation rate and length, however, these seem to be the most impactful host fish on the mussel species present, considering they were the only fish species to be heavily infested. Red shiners and bullhead minnows tended to increase in infestation rate as the length of the fish increased. This is interesting because it would suggest that the buildup of immunity might not occur as often as once thought (Watters and O'Dee, 1996). However, this could be attributed to the release of conglutinates by some freshwater mussel species. Larger minnows would be more capable of foraging on these conglutinates, and thus be more susceptible to glochidia infestation. Blacktail shiners would probably follow a similar trend as the other two minnows, if more individuals were examined. Longear sunfish was not found to be significant, and immunological response probably plays a larger role with this species. Longear sunfish are a long-lived species and an ability to acquire an immunity after repeated exposures would be expected.

Temporal and Spatial Variation in Host Use

This study investigated the temporal and spatial variation in glochidia abundance and host use by East Texas mussel species. However, sampling was not conducted every day throughout the field season, therefore it is not known when fish were initially parasitized, or how long the encystment period truly lasts. *Fusconaia askewi* and *P. riddellii* are considered bradytictic, which is characterized by glochidia release occurring in the spring (Howells et al., 1997). However, there are many exceptions to this trend (Watters and O'Dee, 2000), and both species have been observed to be gravid in July (Howells et al., 1997). Sampling will be somewhat biased based on the mussel community present, whereas localities with high densities of other mussel species relative to species of conservation concern will decrease the chances of finding the target species. However, an attempt was made to bypass this problem by sampling at sites with large mussel beds of known target species.

Glochidia abundance throughout the sampling season was largely seen on three sampling dates. The first large spike occurred in early May and was gone by late May. The second spike occurred in early June and lasted throughout the entire month of June. Although it is not known the exact day on which glochidia were released, it can now be estimated when high peaks of glochidia abundance will occur. This knowledge of known large glochidia releases should be used in future studies when examining glochidia. A laboratory trial testing the host fish found in this study, should capture naturally parasitized fish during either early May or June, to increase the chance of successfully capturing infested fish.

This large temporal study at the Sabine River can also be useful in estimating a release date for each glochidal species identified and an estimated time of encystment. This information can estimate the release date of these species' glochidia by examining the range of dates prior to

finding them. It can also predict how long the glochidia stay encysted by examining the days following encystment in which they are no longer present. *Quadrula mortoni, T. trucata,* and *P. purpuratus* were all identified on only one sampling date, while *P. dombeyanus* was identified on two consecutive sampling dates, and *Q. verrucosa* was identified on two sampling dates a month apart. Based on this information, glochidia release is estimated for *Q. verrucosa* sometime prior to March 8th, *Q. mortoni* sometime between April 23rd and May 14th, *T. truncata* sometime between May 14th and May 28th, *P. purpuratus* sometime between May 28th and June 11th, and *P. dombeyanus* sometime between June 11th and June 25th. It is also estimated that *Q. mortoni, T. trucata,* and *P. purpuratus* have a short encystment time of less than two weeks, while *P. dombeyanus* has an encystment time of about one month, and *Q. verrucosa* has an encystment time of greater than one month. However, considering the small number of glochidia identified for these species, sampling error might have occurred causing a misleading representation of when glochidia were actually released and estimated encystment time.

Fusconaia askewi was found during the entire sampling period in the Sabine River, causing problems in estimating a time of glochidia release for this species. However, there were two large spikes seen in the abundance of *F. askewi* glochidia. These two dates occurred in early May and early June. This suggests that *F. askewi* can release their glochidia throughout the entire spring and summer season, but majority of individuals have timed releases in early May and June. Similar trends were seen in closely related *Fusconaia* species located in an Alabama River (Hagg and Warren, 2003; Culp et al., 2011). The small presence of *F. askewi* seen in early March might suggest this species can overwinter on fish host. Overwintering has been observed in many mussel species (Mishra and Chubb, 1969; Tedla and Fernando, 1969; Dartnall, 1973; Wooten, 1973; Campbell, 1974; Dartnall and Walkey, 1979; Jansen, 1991), but it is assumed to

be attributed to cold temperatures slowing metamorphosis (Watters and O'Dee, 1999), which may have little impact on glochidia in Texas river systems.

Spatial observations were made for host use by *F. askewi* between the Sabine and Neches Rivers. *Fusconaia askewi* was found to infest more species from the Sabine River than the Neches River, however, this is likely a result of examining more species of fish from the Sabine River. The high infestation rates of red shiners, blacktail shiners, and bullhead minnows was similar between both river drainages, providing further evidence that these three species are effective host fish for *F. askewi.*

Morphological Identification of Glochidia

While a morphological identification key to aid in the identification of glochidia was attempted, it was ultimately unsuccessful and abandoned. A compound light microscope failed in accurately measuring glochidia for length, width, and hinge length. An electron microscope would be more efficient in grouping of glochidia based on these features. Once glochidia are encysted in fish tissue, they become difficult or impossible to accurately measure for the creation of a morphological key. Kennedy and Haag (2005) showed the potential success of a morphological key for glochidia of freshwater mussels in an Alabama River. This key was created using glochidia extracted from gravid females, and this is probably the ideal approach as it can accurately measure glochidia while avoiding the attachment of unwanted fish tissue.

Fish Distributions to Predict Potential Fish Host

Jackknife results for modeling with only one of the environmental layers at a time, can reveal if a fish species' distribution explains the distribution of a mussel species. If a fish species layer explains some of the full model of a mussel species, it could be a potential fish host. *Pleurobema riddellii* was found to be naturally encysted on bullhead minnows and red shiners

(Chapter 2). These two fish species were useful in explaining the distribution of *P. riddellii* when modeled alone (47% and 59% respectively). The Maxent results plus the finding of naturally parasitized fish adds weight to the hypothesis that these two species are probably true hosts for *P. riddellii*. Furthermore, blacktail shiners are another potential host, as their distribution, when modeled alone, accounted for 77% of the gain of the full mussel model. Blacktail shiners and red shiners are probably hosts for the same species of mussel, as they are closely related and known to hybridize (Schwartz, 1981; Schonhuth and Mayden, 2010). Increased sampling for *P. riddellii* could reveal glochidia encysted on some of these other fish species.

From Chapter 2, *F. askewi* were found to be naturally infested on 17 fish species, 13 of which were successfully created into fish layers. *Fusconaia askewi* was identified in high infestations on four of these fish (blacktail shiner, bullhead minnow, longear sunfish, and red shiner), all of which when modeled alone accounted for a high percentage of the full model for *F. askewi* (87%, 86%, 96%, and 90% respectively), providing more evidence that these species are likely hosts in natural populations. *Fusconaia askewi* seems like a generalist based on the high number of fish species (17) in which its glochidia were encysted on (Chapter 2). The Maxent results also indicate *F. askewi* as a generalist, as all 23 fish layers when modeled alone accounted for greater than 50% of the mussels' distribution, and 14 of these fish layers accounted for greater than 75% of the mussels distribution. It is interesting that the *F. lananensis* distribution was not strongly associated with the distribution of any fish species. This could be because *F. lananensis* is not in fact a distinct species. Recent evidence suggests that it is closely related to the *F. askewi* and it has been argued that these two are in fact one species (Burlakova et al. 2012).

The other four mussel species have very little or no fish host information, but their models can be used as a method to predict potential fish hosts. *Lampsilis satura* has been shown to use bluegill as a potential host fish in laboratory trials. The Maxent results support bluegill as potential host, as the bluegill layer was able to account for 80% of the distribution for *L. satura*. Many Lampsiline species have evolved an elaborate minnow-like mantle lure to attract host fish (Haag and Warren, 1999), and thus are specialists on piscivorous fish. This also seems to be the case with *L. satura*, as many piscivore fish species (bluegill, largemouth bass, longear sunfish, and white crappie), helped explain the distribution of this mussel. The results for *O. jacksoniana* showed only five fish species that when modeled alone can explain >50% of the gain of the full mussel model for *O. jacksoniana*. There is no other data on fish hosts for *O. jacksoniana*, therefore these five species should be examined for suitability as hosts. Although, it is possible that the true host fish species were not included as layers in this model. No host information is available for *P. amphichaenus*, however Maxent shows relationships between the distribution of many centrarchid species and the distribution of this mussel. It is possible that *P. amphichaenus* uses multiple fish species within this family as fish hosts. Other *Potamilus* species are known to use freshwater drum as a fish host, and this could also be the case with *P. amphichaenus*, as the test gain for freshwater drum when modeled alone is 77%.

Morphological identification keys have had limited success of determining the species of naturally parasitized glochidia on fish. Recently, studies have begun to use molecular identification keys, yet, still very little is known about host use in the wild. Studies examining naturally parasitized fish are usually restricted to one or a few localities, which might be insufficient for a wide-ranging mussel species. Also, many laboratory trials are limited to fish species obtained from a few collection areas. On a broad scale, host species and host use could

differ throughout the range of some mussel species. Wide geographic areas have been examined for the relationship between mussel and fish species richness over the entire community (Vaughn and Taylor, 2000). However, this approach does not explore host suitability or host use. A fine scale approach has examined for some correlation between the distribution of mussel species and their hosts over a small geographic area (Haag and Warren, 1998), yet, some species were positively correlated with host fish and others were negatively correlated. When host fish information is unknown, correlation data might predict potential host fish, but considering some species have a negative correlation with host fish, correlation data should be paired with information from natural infestations or laboratory trials.

CONCLUSIONS

In summary, we were able to successfully molecularly identify glochidia from 8 species of mussel, including two species of conservation concern. These glochidia were identified on wild-caught fish, which provides direct evidence for fish host use in natural conditions. This information is also useful in determining approximate release dates and encystment time for each mussel species identified. Proper management of unionids is not possible without the correct knowledge of fish host use. Relocation efforts for mussels will ultimately be unsuccessful if fish host are absent at the newly relocated site, as long-term recruitment will not be possible. Propagation of species of conservation concern in a laboratory setting is also dependent upon compatibility with proper fish hosts. The life-history data collected throughout this study are crucial for the conservation of this imperiled group.

This study also demonstrates the usefulness of Maxent to aid in the identification of fish hosts when laboratory trials are not available. Maxent was useful in predicting host fish that were shown to carry glochidia of that particular mussel species in the wild. When multiple fish

are found to infest a single mussel species, such as *F. askewi*, Maxent can possibly determine which fish species are the most efficient hosts. It might also be useful in predicting potential host fish for mussel species that have yet to be investigated. Rare mussel species are scarcely abundant in high numbers, therefore, natural infestations of glochidia for such species increase in difficulty to find and identify. Maxent can be used to predict potential fish hosts that strongly contribute to a mussel's distribution, so these species can be tested in the laboratory or heavily sampled in the wild.

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Figure 1. Glochidia abundance found on each sampling date from the Sabine River. Fish were examined for glochidia bimonthly from March to August of 2013. There were two large spikes of glochidia abundance, the first occurring in early May and the second occurring in early June.

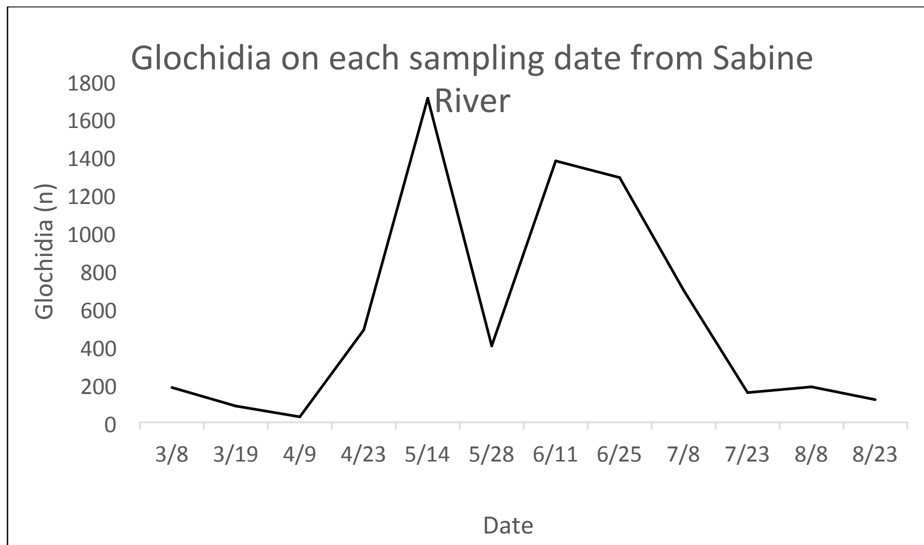

Figure 2. Seasonal change in glochidia diversity in the Sabine River. This information is useful in determining the approximate release date and encystment length for each species identified. *Fusconaia askewi* was found during the entire sampling season and was found in two large spikes, possibly coinciding with two major release events.

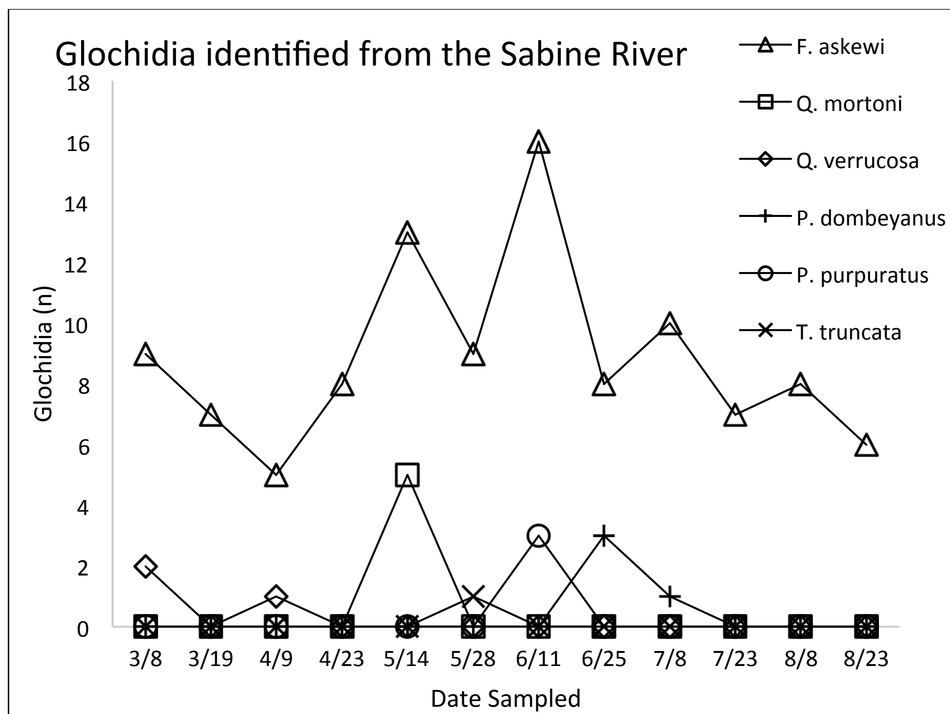

Figure 3. Data for all 23 species found to be infested with glochidia from the Sabine River during the bimonthly sampling from March 2013 to August 2013. The ideal fish host will be situated in the upper right corner on this 3d plot, as it would be highly abundant, heavily infested, and infested at high rates.

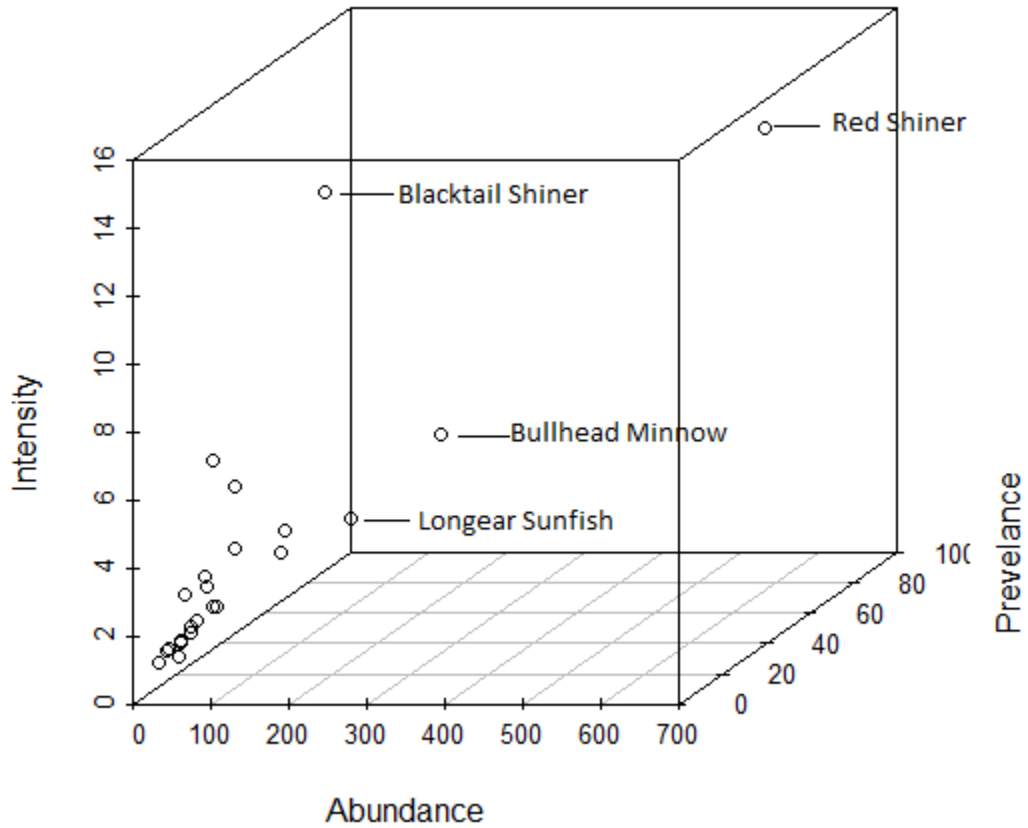

Table 1: Fish species captured from Sabine River found to be encysted with glochidia. Fish were examined for glochidia bimonthly from March to August of 2013.

	Fish Species	Caught (n)	Infested (n)	Infested (%)	% Infested with ≥20 glochidia	Glochidia (n)
Clupeidae	*Dorosoma cepedianum*	25	6	24.0	-	16
Cyprinidae	*Cyprinella lutrensis*	632	404	63.9	22.0	5700
	Cyprinella venusta	46	33	71.7	18.2	392
	Hybopsis amnis	8	1	12.5	-	1
	Notemigonus crysoleucas	9	3	33.3	-	17
	Notropis texanus	3	2	66.7	-	3
	Pimephales promelas	7	1	14.3	-	1
	Pimephales vigilax	356	49	13.8	4.1	358
Ictaluridae	*Ictalurus punctatus*	17	7	41.2	-	32
	Noturus nocturnus	34	5	14.7	-	8
Escocidae	*Esox americanus*	3	1	33.3	-	2
Aphredoderidae	*Aphredoderus sayanus*	19	1	5.3	-	1
Atherinidae	*Labidesthes sicculus*	1	1	100.0	-	1
Poeciliidae	*Gambusia affinis*	36	3	8.3	-	3
Fundulidae	*Fundulus notatus*	39	4	10.3	-	11
Centrarchidae	*Lepomis macrochirus*	41	9	22.0	-	17
	Lepomis megalotis	147	25	17.0	4.0	109
	Micropterus punctulatus	15	5	33.3	-	7
	Micropterus salmoides	11	2	18.2	-	2
Percidae	*Etheostoma asprigene*	12	3	25.0	-	4
	Etheostoma gracile	5	1	20.0	-	1
	Percina sciera	31	11	35.5	-	33
Sciaenidae	*Aplodinotus grunniens*	4	1	25.0	-	1

Table 2: Fish species captured from Neches River found to be encysted with glochidia. Fish were examined for glochidia once in June and once in August of 2013.

	Fish species	Caught (n)	Infested (n)	Infested (%)	% Infested with ≥20 glochidia	Glochidia (n)
Clupeidae	*Dorosoma cepedianum*	2	0	0	-	0
Cyprinidae	*Cyprinella lutrensis*	59	48	81.4	20.8	540
	Cyprinella venusta	24	18	75	5.6	212
	Cyprinus carpio	1	0	0	-	0
	Macrhybopsis hyostoma	2	0	0	-	0
	Pimephales vigilax	38	17	44.7	5.9	64
Ictaluridae	*Ictalurus punctatus*	1	1	100	-	1
	Noturus nocturnus	1	0	0	-	0
Centrarchidae	*Lepomis cyanellus*	2	0	0	-	0
	Lepomis macrochirus	4	2	50	-	1
	Lepomis megalotis	5	1	20	-	3
Percidae	*Etheostoma asprigene*	2	0	0	-	0
Sciaenidae	*Aplodinotus grunniens*	1	1	100	-	3

Table 3: Environmental layers for 23 fish species that were incorporated into creating mussel models. All 23 fish species had large AUC values (>0.75), and 17 of the 23 fish species were found to be naturally encysted with glochidia in the wild.

	AUC	Number of Locations	Encysted with Glochidia in Wild
Blackstripe Topminnow	0.9213	7	Y
Blacktail Shiner	0.9254	11	Y
Bluegill	0.8934	14	Y
Bullhead Minnow	0.919	9	Y
Channel Catfish	0.8976	7	Y
Dollar Sunfish	0.8253	6	N
Dusky Darter	0.8948	5	Y
Freckled Madtom	0.9172	9	Y
Freshwater Drum	0.9538	6	Y
Gizzard Shad	0.9201	8	Y
Green Sunfish	0.8966	6	N
Largemouth Bass	0.8393	7	Y
Longear Sunfish	0.879	12	Y
Orangespotted Sunfish	0.8645	5	N
Pirate Perch	0.8724	6	Y
Red Shiner	0.9341	11	Y
Ribbon Shiner	0.8934	7	N
Smallmouth Buffalo	0.985	5	N
Spotted Bass	0.8504	10	Y
Warmouth	0.8221	5	N
Weed Shiner	0.9103	7	Y
Western Mosquito Fish	0.8996	5	Y
White Crappie	0.9031	5	N

Table 4t: Percent test gain attributed to each mussel model with the use of only one fish layer. High percentage indicates a correlation between the distribution of a fish species and the corresponding mussel species. This correlation can be used to investigate potential fish hosts.

	Louisiana Pigtoe	Sandbank Pocketbook	Southern Hickorynut	Texas Heelsplitter	Texas Pigtoe	Triangle Pigtoe
Blackstripe Topminnow	65.57	55.86	56.35	26.52	64.90*	28.96
Blacktail Shiner	77.53	102.46	67.11	85.80	87.44**	29.21
Bluegill	57.50	80.01	38.35	103.88	92.55*	26.15
Bullhead Minnow	47.22*	69.78	30.80	95.66	86.37**	18.69
Channel Catfish	51.02	65.99	35.09	83.66	78.40*	21.68
Dollar Sunfish	35.50	47.69	28.81	97.27	65.88	27.33
Dusky Darter	75.45	97.88	73.06	95.58	92.57*	27.98
Freckled Madtom	49.30	68.95	33.65	92.21	79.43*	19.70
Freshwater Drum	61.16	85.66	47.76	64.32	77.03	33.23
Gizzard Shad	60.59	85.06	47.77	66.82	78.76	26.64
Green Sunfish	27.45	37.96	28.84	47.78	56.02	23.84
Largemouth Bass	61.78	79.57	42.93	54.81	84.54	24.48
Longear Sunfish	63.57	95.81	47.96	107.98	96.29**	28.60
Orangespotted Sunfish	38.69	40.64	13.64	83.20	70.82	15.59
Pirate Perch	42.16	39.24	32.52	25.58	62.96	28.95
Red Shiner	59.33*	81.32	55.43	98.97	89.60**	34.00
Ribbon Shiner	36.19	50.20	23.94	83.52	68.22	17.56
Smallmouth Buffalo	59.41	78.43	44.33	92.36	82.27	20.09
Spotted Bass	63.56	87.83	49.15	99.55	95.16*	23.42
Warmouth	32.64	45.74	38.93	25.98	59.52	34.17
Weed Shiner	69.06	63.02	60.13	28.99	65.49*	30.99
Western Mosquito Fish	17.04	35.01	25.40	66.22	62.14*	33.11
White Crappie	59.91	80.47	43.16	53.27	83.69	26.71

*Fish species was naturally parasitized with corresponding mussel species
**Fish species was heavily parasitized in the wild